Pertanika Journal of

# TROPICAL AGRICULTURAL SCIENCE

JTAS

VOL. 47 (4) NOV. 2024

PERTANIKA JOURNALS

A scientific journal published by Universiti Putra Malaysia Press

*Pertanika* Journal of Tropical Agricultural Science
*Pertanika* Journal of Tropical Agricultural Science

# *PERTANIKA* JOURNAL OF TROPICAL AGRICULTURAL SCIENCE

## About the Journal

### *Overview*

*Pertanika* Journal of Tropical Agricultural Science is an official journal of Universiti Putra Malaysia. It is an open-access online scientific journal. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognised internationally as the leading peer-reviewed interdisciplinary journal devoted to the publication of original papers, it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields.

*Pertanika* Journal of Tropical Agricultural Science is a **quarterly** (*February*, *May*, *August*, and *November*) periodical that considers for publication original articles as per its scope. The journal publishes in **English** and it is open for submission by authors from all over the world.

The journal is available world-wide.

### *Aims and scope*

*Pertanika* Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance, and veterinary medicine.

### *History*

*Pertanika* was founded in 1978. Currently, as an interdisciplinary journal of agriculture, the revamped journal, *Pertanika* Journal of Tropical Agricultural Science now focuses on tropical agricultural research and its related fields.

### *Vision*

To publish journals of international repute.

### *Mission*

Our goal is to bring the highest quality research to the widest possible audience.

### *Quality*

We aim for excellence, sustained by a responsible and professional approach to journal publishing. Submissions are guaranteed to receive a decision within 90 days. The elapsed time from submission to publication for the articles averages 180 days. We are working towards decreasing the processing time with the help of our editors and the reviewers.

### *Abstracting and indexing of Pertanika*

*Pertanika* Journal of Tropical Agricultural Science is now over 45 years old; this accumulated knowledge has resulted in *Pertanika* Journal of Tropical Agricultural Science being abstracted and indexed in SCOPUS (Elsevier), Clarivate Web of Science (ESCI), EBSCO, DOAJ, Agricola, ASEAN CITATION INDEX, ISC, Microsoft Academic, Google Scholar, National Agricultural Science (NAL), and MyCite.

### *Citing journal articles*

The abbreviation for *Pertanika* Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci*.

### *Publication policy*

*Pertanika* policy prohibits an author from submitting the same manuscript for concurrent consideration by two or more publications. It prohibits as well publication of any manuscript that has already been published

either in whole or substantial part elsewhere. It also does not permit publication of manuscript that has been published in full in proceedings.

### *Code of Ethics*

The *Pertanika* journals and Universiti Putra Malaysia take seriously the responsibility of all its journal publications to reflect the highest publication ethics. Thus, all journals and journal editors are expected to abide by the journal's codes of ethics. Refer to *Pertanika*'s **Code of Ethics** for full details, available on the official website of *Pertanika*.

### *Originality*

The author must ensure that when a manuscript is submitted to *Pertanika*, the manuscript must be an original work. The author should check the manuscript for any possible plagiarism using any program such as Turn-It-In or any other software before submitting the manuscripts to the *Pertanika* Editorial Office, Journal Division.

All submitted manuscripts must be in the journal's acceptable **similarity index range**:
**≤ 20%** – *PASS*; **> 20%** – *REJECT*.

### *International Standard Serial Number (ISSN)*

An ISSN is an 8-digit code used to identify periodicals such as journals of all kinds and on all media–print and electronic. All *Pertanika* journals have an e-ISSN.

*Pertanika* Journal of Tropical Agricultural Science: e-ISSN 2231-8542 (Online).

### *Lag time*

A decision on acceptance or rejection of a manuscript is expected within 90 days (average). The elapsed time from submission to publication for the articles averages 180 days.

### *Authorship*

Authors are not permitted to add or remove any names from the authorship provided at the time of initial submission without the consent of the journal's Chief Executive Editor.

### *Manuscript preparation*

For manuscript preparation, authors may refer to *Pertanika*'s **INSTRUCTION TO AUTHORS**, available on the official website of *Pertanika*.

### *Editorial process*

Authors who complete any submission are notified with an acknowledgement containing a manuscript ID on receipt of a manuscript, and upon the editorial decision regarding publication.

*Pertanika follows* a double-**blind peer review** process. Manuscripts deemed suitable for publication are sent to reviewers. Authors are encouraged to suggest names of at least 3 potential reviewers at the time of submission of their manuscripts to *Pertanika,* but the editors will make the final selection and are not, however, bound by these suggestions.

Notification of the editorial decision is usually provided within 90 days from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

### *The journal's peer review*

In the peer review process, 2 or 3 referees independently evaluate the scientific quality of the submitted manuscripts. At least 2 referee reports are required to help make a decision.

Peer reviewers are experts chosen by journal editors to provide written assessment of the **strengths** and **weaknesses** of written research, with the aim of improving the reporting of research and identifying the most appropriate and highest quality material for the journal.


*Pertanika* Journal of Tropical Agricultural Science
*Pertanika* Journal of Tropical Agricultural Science

*Pertanika* Journal of Tropical Agricultural Science
*Pertanika* Journal of Tropical Agricultural Science

## *Operating and review process*

What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are 7 steps to the editorial review process:

1. The journal's Chief Executive Editor and the Editor-in-Chief examine the paper to determine whether it is relevance to journal needs in terms of novelty, impact, design, procedure, language as well as presentation and allow it to proceed to the reviewing process. If not appropriate, the manuscript is rejected outright and the author is informed.

2. The Chief Executive Editor sends the article-identifying information having been removed, to 2 or 3 reviewers. They are specialists in the subject matter of the article. The Chief Executive Editor requests that they complete the review within 3 weeks.

   Comments to authors are about the appropriateness and adequacy of the theoretical or conceptual framework, literature review, method, results and discussion, and conclusions. Reviewers often include suggestions for strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the research field.

3. The Editor-in-Chief examines the review reports and decides whether to accept or reject the manuscript, invite the authors to revise and resubmit the manuscript, or seek additional review reports. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the authors) are forwarded to the authors. If a revision is indicated, the editor provides guidelines to the authors for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.

4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors return a revised version of the paper to the Chief Executive Editor along with specific information describing how they have answered' the concerns of the reviewers and the editor, usually in a tabular form. The authors may also submit a rebuttal if there is a need especially when the authors disagree with certain comments provided by reviewers.

5. The Chief Executive Editor sends the revised manuscript out for re-review. Typically, at least 1 of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the Editor-in-Chief examines their comments and decides whether the manuscript is ready to be published, needs another round of revisions, or should be rejected. If the decision is to accept, the Chief Executive Editor is notified.

7. The Chief Executive Editor reserves the final right to accept or reject any material for publication, if the processing of a particular manuscript is deemed not to be in compliance with the S.O.P. of *Pertanika.* An acceptance notification is sent to all the authors.

   The editorial office ensures that the manuscript adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any minor queries by the editorial office. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, **only essential changes are accepted**. Finally, the manuscript appears in the pages of the journal and is posted on-line.

Pertanika Journal of

# TROPICAL AGRICULTURAL SCIENCE

Vol. 47 (4) Nov. 2024

PERTANIKA JOURNALS

A scientific journal published by Universiti Putra Malaysia Press

# PJTAS

***Pertanika* Journal of Tropical Agricultural Science**

AN INTERNATIONAL PEER-REVIEWED JOURNAL





**EDITORIAL OFFICE**

**JOURNAL DIVISION**

Putra Science Park

1st Floor, IDEA Tower II

UPM-MTDC Technology Centre

Universiti Putra Malaysia

43400 Serdang, Selangor Malaysia.

**Gen Enquiry**

Tel. No: +603 9769 1622 | 1616

E-mail:

executive_editor.pertanika@upm.edu.my

URL: www.journals-jd.upm.edu.my

**PUBLISHER**

**UPM PRESS**

Universiti Putra Malaysia

43400 UPM, Serdang, Selangor, Malaysia.

Tel: +603 9769 8851

E-mail: penerbit@putra.upm.edu.my

URL: http://penerbit.upm.edu.my





**ABSTRACTING AND INDEXING OF PERTANIKA JOURNALS**

The journal is indexed in SCOPUS (Elsevier), Clarivate-Emerging Sources Citation Index (ESCI), BIOSIS, National Agricultural Science (NAL), Google Scholar, MyCite, ISC. In addition, Pertanika JSSH is recipient of "CREAM" Award conferred by Ministry of Higher Education (MoHE), Malaysia.

**Pertanika Journal of Tropical Agricultural Science**
**Vol. 47 (4) Nov. 2024**

## Contents

**Selected papers from the 11th International Conference on Multidisciplinary Research (2023)**
Guest Editor: Enis Nadia Md Yusof

# Foreword

Welcome to the fourth issue of 2024 for the Pertanika Journal of Tropical Agricultural Science (PTAS)!

PJTAS is an open-access journal for studies in Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university for the benefit of the world-wide science community.

This issue contains 25 articles: four review articles; one short communication; one case study; and the rest are regular articles. The authors of these articles come from different countries namely Bangladesh, Indonesia, Malaysia, Nigeria and Vietnam.

Ratih Hardiyanti and her teammates from Universitas Gadjah Mada have assessed the extraction methods of the noni fruit (*Morinda citrifolia* L.) and ensured appropriate method selection for the isolation of optimal bioactive ingredients. They found out that noni fruit contains phytochemical components that have multiple health benefits, which include polyphenols and flavonoids. In addition, there are several extraction techniques available that can generate optimally bioactive extracts. The separation technique for noni fruit was solid-liquid extraction or leaching. The detailed information of this article is available on page 1139.

The regular article entitled "*In-vivo* Toxicity Assessment of the Garlic Juice Extract (*Allium sativum*) in Juvenile Hybrid Grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*)" evaluated the toxicity of garlic juice extract in juvenile hybrid grouper via bath and oral administration. A total of 280 fish, each with an average weight of 20 ± 5 g, were evenly distributed among 28 glass aquaria. The fish were immersed in freshly prepared garlic juice extracts at 0, 500, 600, 700, 800, 900, and 1,000 ppm concentrations. Meanwhile, pellets containing 0, 20, 40, 60, 80, and 100% garlic juice extract were administered for oral exposure. The median lethal concentration of garlic juice extract following bath immersion was recorded at 993.11 ppm after 96 hr. Besides, there was no mortality in all groups exposed to garlic juice extract orally, indicating that the extract has a shallow effect on juvenile hybrid groupers when ingested. Further details of this study are found on page 1379.

A selected article entitled "The Effect of Biofertilizer Dose on Growth and Yield of Four Maize Varieties in Indonesia" determined the effect of biofertilizer application in liquid and granule form with several doses on the growth and yield of four maize varieties. The study was carried out using nested randomized complete block design with two factors, i.e., 7 biofertilizers (two formulas, i.e., liquid and granule with 3 levels of dosage) and 4 maize varieties. The results showed that the application of biofertilizers affects the growth and yield of several maize varieties. Granular

biofertilizer at a dose of 150 g/plant showed the best growth and yield observed variables, and Bisi 99 showed the best performance compared with other maize varieties. Full information of this study is presented on page 1391.

In the last 12 months, of all the manuscripts peer-reviewed, 40% were accepted. This seems to be the trend in PJTAS.

We anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

All the papers published in this edition underwent Pertanika's stringent peer-review process involving a minimum of two reviewers comprising internal as well as external referees. This was to ensure that the quality of the papers justified the high ranking of the journal, which is renowned as a heavily-cited journal not only by authors and researchers in Malaysia but by those in other countries around the world as well.

We would also like to express our gratitude to all the contributors, namely the authors, reviewers, Editor-in-Chief and Editorial Board Members of PJTAS, who have made this issue possible.

PJTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

**Chief Executive Editor**
**Mohd Sapuan Salit**
executive_editor.pertanika@upm.edu.my

Pertanika J. Trop. Agric. Sci. 47 (4): 1077 - 1086 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Plant Description, Growth, and Flowering of Two Indonesian Jasmines

**Mega Shintia, Krisantini* and Ani Kurniawati**

*Department of Agronomy and Horticulture, Bogor Agricultural University, Jalan Meranti, Darmaga Campus, Bogor 16680, Indonesia*

## ABSTRACT

Jasmine flowers **(***Jasminum sambac***)** are very popular in Indonesia and Asia because of their strong sweet fragrance. Jasmine flowers have been used to decorate weddings, houses of worship, and festivals and are included in aromatic products like candles, perfumes, soaps, and lotions. Despite its popularity, studies on morphology, growth, and flowering characteristics of various jasmine species are lacking. Our study aims to describe the morphology and flowering of jasmine cultivars from a relatively humid environment, Java Island (Emprit Bandar Arum), and a relatively drier environment, Madura Island (Ratoh Ebuh), Indonesia. Each cultivar has seven blocks of four plants each block. The study was conducted at Cikabayan, West Java, Indonesia, from April 2022 to February 2023. Java and Madura's jasmine have morphological similarities in their stem shape and color, leaf type, shape and arrangement, calyx structure, petal shape, and flower color. The two jasmine species differ in leaf size, leaf tip shape, stomatal size and density, and duration from floral bud initiation to anthesis. Madura jasmines produced about 20% more flowers than Java jasmines. Understanding the morphology and flowering of different types of jasmine is important to correctly identify the jasmine cultivars to determine the peak flowering season and flower production. A list of characters that can potentially be used for future studies of the two jasmine cultivars was described. The results of this study would benefit commercial growers in choosing the right type/cultivar to grow and predicting the time to harvest the flowers in a particular region.

*Keywords*: Floral morphology, *Jasminum sambac*, Java, Madura

ARTICLE INFO

*Article history:*
Received: 18 June 2023
Accepted: 21 December 2023
Published: 27 September 2024

DOI: https://doi.org/10.47836/pjtas.47.4.01

*E-mail addresses*:
megashintia@apps.ipb.ac.id (Mega Shintia)
krisantini@apps.ipb.ac.id (Krisantini)
ani_kurniawati@apps.ipb.ac.id (Ani Kurniawati)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

## INTRODUCTION

The genus *Jasminum* (Oleaceae) includes approximately 200 species distributed in the tropical and subtropical regions of the world (Green & Miller, 2009; Jeyarani et al., 2018). Jasmines are characterized by highly fragrant flowers, especially *Jasminum grandiflorum, Jasminum sambac, and Jasminum auriculatum,* the three main species currently grown commercially for jasmine oil production (Yohanan et al., 2020). The genus *Jasminum* is known to have 40 species cultivated in India: among them, 20 species are cultivated in south India (Bhattacharjee, 1980; Chaitanya et al., 2020). Jasmine flowers have been used to decorate houses of worship, religious ceremonies, and festivals: flowers can be placed in water as part of the ritual worship of Hinduism (Ali & Sosa, 2015; Demole et al., 1962). Jasmine flower extracts are raw materials used in perfume production and as a drink made by blending jasmine flowers with loose-leaf green tea (Ali & Sosa, 2015; Atal & Kapur, 1982). In addition, jasmine flower extracts are effective as wound healers (Nayak & Mohan, 2007) and have antimicrobial activities (Shekhar & Prasad, 2015). In Indonesia, the production center of the jasmines is concentrated in Central Java (Hartati & Darsana, 2015) and Madura Island. Madura has a production area of 50 ha in Bangkalan Regency (Tamami, 2012). Emprit Bandar Arum is a *J. sambac* cultivar from Central Java, bred by Sri Rustini. It has been certified by the Ministry of Agriculture with the Decree of the Minister of Agriculture Number 6036/Kpts/SR.120/12/2012 (Musalamah et al., 2019). Emprit Bandar Arum flowers are white in color and highly fragrant. They are widely used for tea mixtures, export, and as raw material for the perfume industry (Palupi et al., 2019). Ratoh Ebuh is another *J. sambac* originating from Buluh Atas Village, Bangkalan Regency (M. Sari & Tamami, 2020). Like Emprit bandar Arum, Ratoh Ebuh has white and fragrant flowers that are longer lasting than the jasmines from Central Java and are well adapted to low to medium elevations (Marfirani et al*.,* 2014). Ratoh Ebuh cultivar was registered by the Decree of the Minister of Agriculture Number: 515/Kpts/SR.120/12/2005 on December 26, 2005.

Despite the high demand for jasmine flowers, studies on the description, morphology, growth, and flowering pattern in different regions still need to be completed. Understanding the morphology and flowering of different types of jasmine has several implications for commercial production. It helps growers choose the right type/cultivar to grow and adapt to the right environment to maximize plant growth and flowering. The knowledge of jasmine morphology and flowering would help select the market suitability, e.g., flower bouquets, perfumes, or cosmetics, as the flower quality varies with cultivars. Jasmine production in Indonesia is primarily traditional, with little input and maintenance, as jasmine is considered an easy crop to grow. The objective of the present study is to provide the plant description and leaf morphology and study the growth and flowering of a

jasmine cultivar from Central Java (Emprit Bandar Arum) and Madura Island (Ratoh Ebuh). Java and Madura have different microclimates: Java (the western part of Indonesia) is generally more humid and has higher yearly rainfall than Madura Island. The results of this study will provide helpful information for the growers to identify and differentiate the two cultivars and to understand their growth habits and flowering for commercial production.

## MATERIALS AND METHODS

The study was conducted at the Cikabayan experimental field, Department of Agronomy and Horticulture, IPB University, from April 2022 to February 2023. The location has an altitude of 234 m above mean sea level, with an average monthly temperature of 29°C, average rainfall of 187 mm/month, and humidity of 55–95%. The plant materials are rooted cuttings with four nodes and four leaves per cutting, grown in 10 L polybags with media mixtures consisting of 20% (v/v) soil, 10% (v/v) husk charcoal, 20% (v/v) cocopeat, and composted 50% (v/v) bamboo leaves. Each plant was supplied with 2 g/L Osmocote Plus 8-9M (N:P:K = 17:11:10, Netherlands) at planting.

### Treatments, Data Collection, and Analysis

The study was organized in a completely randomized block design with the jasmine cultivar as a single factor: each cultivar had seven blocks, which consisted of 4 plants per block, totaling 224 plants. The two jasmine cultivars were evaluated for the leaf, stem, and flower morphological traits, including stem shape, stem color, leaf shape and type, leaf arrangement, leaf tip, leaf bases, leaf shape, peduncle color, calyx structure, calyx color, petal color, and petal shape according to Clarke and Lee (2019). The morphological traits according Clarke and Lee (2019) are summarized as follows: leaf shape is either simple or compound; leaf arrangements are alternate, opposite, or whorled; leaf tips are obtuse, acute, aristate, acuminate, obtuse, truncate, retuse, or emarginate; leaf bases are attenuate, cuneate, obtuse, truncate, cordate, sagittate, hastate, oblique, or peltate; calyx (sepals) can be whole or partly united; corolla (petals) can be actinomorphic or zygomorphic.

Quantitative data were collected on the plant height, number of leaves, branches, and flower production, measured every two weeks between the first week of October 2022 and the end of February 2023. Plant height was measured on the main stem, from the stem base to the tip of the growing point, using a ruler. Leaf number is calculated based on the total number of mature, fully expanding leaves per plant. The branch number is calculated based on the total number of primary and secondary branches per plant. Plant height, leaf number, and branch number measurements were conducted on all plants (7 blocks of 4 plants per block). Leaf width and length were measured on one fully expanded leaf per plant and three randomly sampled plants per block. Flower production is the total number of fully opened flowers per plant, which were counted twice a week and then added for the duration of the study

(20 weeks). The average and standard error of all quantitative were calculated using Microsoft Excel.

Leaf anatomy was studied by measuring leaf thickness, stomatal density, and stomatal size using microscope Olympus CX 23 (Japan) with 100x magnification. Leaf thickness measurement was conducted on a mature, fully expanded leaf collected from the third node from the apex at 12 weeks after transplanting. Leaves were hand-sectioned using a sharp razor blade (Gillette, Germany). The stomatal observation was conducted to determine their number, density, and size. Measurement of stomatal density used a replica method, i.e., a technique to print the epidermal part of the leaf using transparent nail polish (D. P. Sari & Harlita, 2018). Clear nail polish was painted on the abaxial leaf, avoiding the veins to examine the stomata. Once the nail polish was dry, clear cellophane tape was placed on top of the polish and lifted off the leaf to have the replica of the leaf on the tape. The tape was placed directly on a microscope slide and observed under 40× magnification (Heyneke et al., 2013). The number, density, and area of stomata were calculated using Image J software (version 1.54). Data on the number of stomatal, stomatal density, and stomatal size were analyzed using Microsoft Excel.

## RESULTS AND DISCUSSION

### Plant Description

The Java jasmine (Emprit Bandar Arum) and Madura jasmine (Ratoh Ebuh) have morphological similarities in their stem shape and color, leaf type, shape and arrangement, calyx structure, petal shape, and flower color. Both jasmines have woody, cylindrical stem shapes: the young stems are green, which turns brown when matured. Both have simple leaves and green leaf color and entire leaf margins (Figures 1C and 1G). Both have partly united calyx with light green peduncles (Figures 1C and 1D). The flowers are actinomorphic with white ovate petals that consist of a single layer with a similar diameter size (25–40 mm) (Figures 1A and 1E)

The two jasmines differ in the leaf tip: the leaf tip of the Java jasmine is acute or less pointed (Figure 1C), whereas the Madura jasmine has an acuminated (pointed) leaf tip (Figure 1G). Both jasmines have a cordate leaf base (Figures 1B and 1E).

### Leaf Anatomy

The leaf size of the two cultivars differs, with the leaf lengths of Java jasmine being 8.2–9.9 cm and Madura jasmine 5.6–6.8 cm; the leaf width of Java jasmine 4.5–6.2 cm, and Madura jasmine 2.2–3.2 cm (Figures 1C and 1F). Leaf size varies with genotypes and cultivars: cell size and number are the dominant factors determining leaf area (Hu et al., 2020). The jasmine cultivars have a similar leaf thickness of around 185 µm (Table 1). Both jasmine cultivars have paracytic stomata, i.e., a stomatal type in which the two subsidiary cells are parallel to the long axis of the guard cells (Figure 2). However, the Madura jasmine has a higher stomatal


*Figure 1*. Morphology of a fully opened flower (A), tepal (calyx) (B), a mature leaf (C), and growth habit (D) of Java jasmine (*Jasminum sambac* Emprit Bandar Arum); a fully opened flower (E), tepal (calyx) (F), a mature leaf (G), and growth habit (H) of Madura jasmine (*Jasminum sambac* Ratoh Ebuh)

Table 1
*Leaf thickness and stomatal characteristics of Java and Madura jasmines*

| Genotypes | Stomatal density ($mm^2$) | Stomatal length (µm) | Stomatal width (µm) | Leaf thickness (µm) | Leaf chlorophyll content (mg/g) |
|---|---|---|---|---|---|
| Java jasmine (Emprit Bandar Arum) | 328.43 ± 31.70 | 32.08 ± 4.05 | 21.04 ± 2.66 | 185.05 ± 21.20 | 1.61 ± 0.23 |
| Madura jasmine (Ratoh Ebuh) | 377.07 ± 34.70 | 27.29 ± 2.50 | 23.75 ± 2.48 | 186.80 ± 7.00 | 1.67 ± 0.15 |

density (377.07 $mm^2$) than Emprit Bandar Arum (328.43 $mm^2$). The stomata of the Java jasmine are slightly longer than the Madura jasmine, but the stomatal width is similar (Table 1). Leaf thickness can vary with genotypes within the same species (Coneva & Chitwood, 2018), affecting transpiration and water uptake (Afzal et al., 2017). Genotypes with thick leaves have been reported to retain water during periods of water stress (Coneva & Chitwood, 2018).

In leaves with the same thickness, a larger leaf surface can capture more light energy for photosynthesis and leaves with more photosynthetic pigments can absorb more light energy (Li & Kubota, 2009; Nguyen et al., 2019). Leaves are crucial photosynthetic organs, and the carbon produced from photosynthesis provides energy for plant growth and development. Stomatal size and density are highly affected by the environment, including light intensity (Haworth et al., 2014, 2023), water availability (Haworth et al., 2023; Heyneke et al., 2013), salinity (Haworth et al., 2023; Shabala et al., 2013),

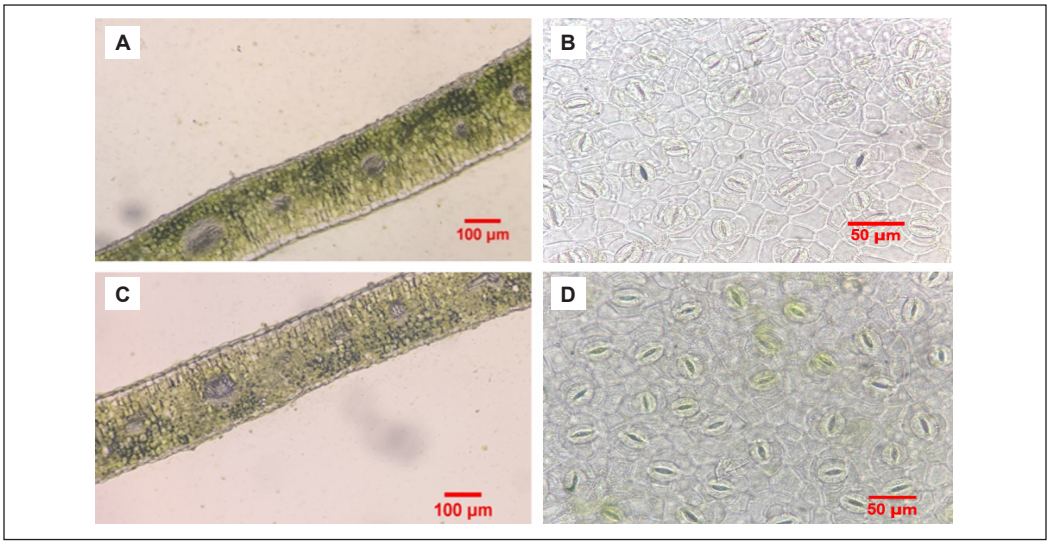



*Figure 2*. Leaf cross-section (A) and stomata (B) of Java jasmine (*Jasminum sambac* Emprit *Bandar* Arum); leaf cross-section (C) and stomata (D) Madura jasmine (*Jasminum sambac* Ratoh Ebuh)

and temperatures (Beerling et al., 1993; Haworth et al., 2023) as these factors affect cell expansion and cell formation. Plants grown in a dry environment with high light intensity have been reported to have many small stomata compared to those grown in cooler and shaded environments (Bertolino et al., 2019). This statement agrees with our finding: Ratoh Ebuh from Madura, which has a drier climate compared to Central Java, has a higher stomatal density (377 per $mm^2$) than Emprit Bandar Arum (328 per $mm^2$).

## Growth and Flowering

The highest growth parameters, plant height, number of branches, and number of leaves were found in Madura jasmine. The Madura jasmines have more branches (Figure 3B) and consequently more leaves (Figure 3C) than the Java jasmine. In terms of growth habits, Madura jasmine (Figure 1F) tends to be shorter and has more branches than Java jasmine (Figure 1C), so the plants look denser.

Java jasmine flowers have fewer total inflorescence (35) than Madura jasmine (45; Table 2). For fresh flower markets, which determine the flower value based on the flower weight, heavier flowers could be obtained from the Madura jasmines, as both cultivars have a similar flower weight of around 200 mg (data not presented). Both jasmine cultivars have their peak flowering in October (week 0) and January (week 10): the Madura jasmines produced more flowers than Java jasmines (Figure 3D). The duration of Madura jasmines from floral bud initiation to anthesis was slightly shorter (less than 14 days) compared to the Java jasmine, which can take more than 14 days.

Understanding the morphology and flowering of different types of jasmine has several implications for commercial production: it helps growers choose the

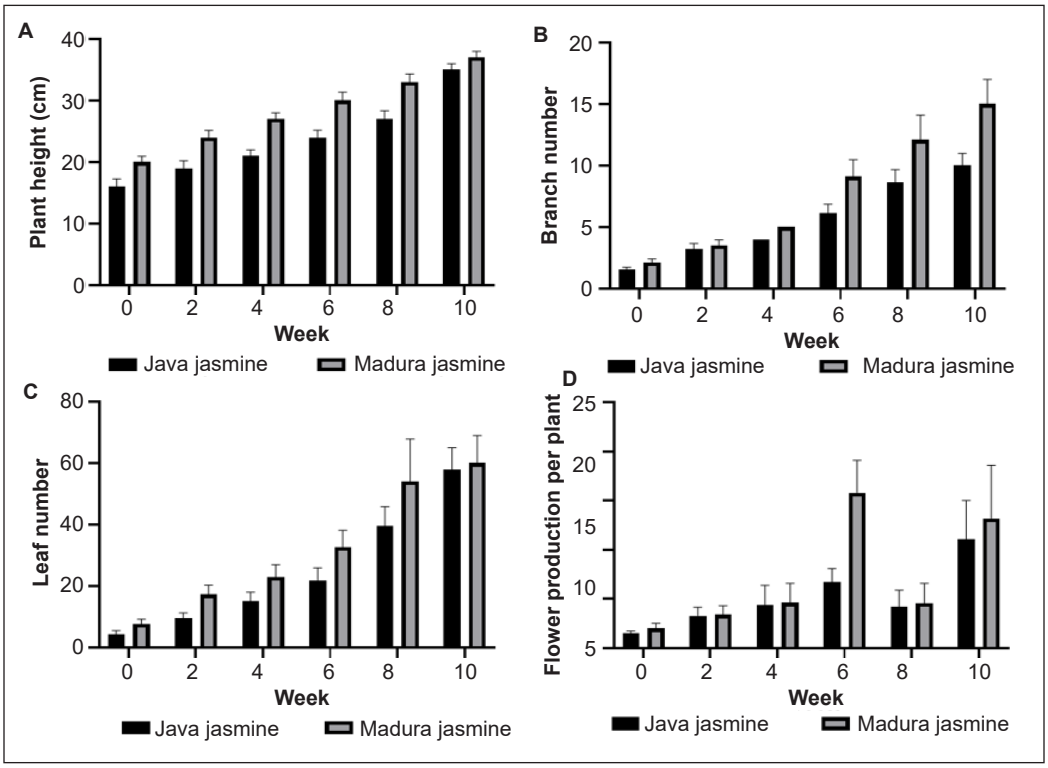



*Figure 3*. Vegetative growth and flower production of Java and Madura jasmines: plant height (A), branch number (B), leaf number (C), and flower production per plant (D)
*Note*. Data are averages ± standard errors

Table 2
*Flowering characteristics of Java and Madura jasmines*

| Genotypes | Time of flowering | Total number of inflorescence per plant* | Number of flowers per inflorescence |
|---|---|---|---|
| Java jasmine (Emprit Bandar Arum) | October, November, December, January, February | 35 | 2-5 |
| Madura jasmine (Ratoh Ebuh) | October, November, December, January, February | 45 | 1-6 |

**Note*. Total inflorescence per plant from October 2022–February 2023

right type/cultivar to grow to determine the peak flowering production and adapt the right environment to maximize plant growth and flowering. The knowledge of jasmine morphology and flowering would help select the market suitability, e.g., flower bouquets, perfumes, or cosmetics, as the flower quality varies with cultivars. Short plant postures with many branches are more suitable as potted ornamental plants than plants that tend to grow tall. Combining knowledge of jasmine morphology and flowering with proper cultivation techniques and suitable environmental settings can result in

jasmines having higher quality flowers and maximum yields, thus increasing profits for the growers.

## CONCLUSION

Java and Madura jasmine have morphological similarities in their stem shape and color, leaf type, shape and arrangement, calyx structure, petal shape, and flower color. The two species differ in the leaf size, the shape of the leaf tip, the stomatal size and density, and the duration from floral bud initiation to anthesis. Java jasmines have larger leaves, less pointed leaf tips, larger stomata, and lower stomatal density than the Madura jasmines. Madura jasmines produced about 20% more flowers than Java jasmines in the West Java environment.

## ACKNOWLEDGEMENTS

The authors thanked the Department of Agronomy and Horticulture, Faculty of Agriculture, IPB University, Bogor, Indonesia, for the facilities and support provided for the study. The authors received no specific funding to support this research work. It was self-funded by the authors.

## REFERENCES

Afzal, A., Duiker, S. W., & Watson, J. E. (2017). Leaf thickness to predict plant water status. *Biosystems Engineering*, *156*, 148-156. https://doi.org/10.1016/j.biosystemseng.2017.01.011

Ali, J. K., & Sosa, A. A. (2015). Anatomical study of some characters in certain species of genus *Jasminum* L. growing in Iraq. *International Journal of Science and Research*, *5*(10), 1137-1140. https://doi.org/10.21275/ART20161966

Atal, C. K., & Kapur, B. M. (Eds.) (1982). *Cultivation and utilization of medicinal plants*. Council of Scientific and Industrial Research.

Beerling, D. J., Chaloner, W. G., Huntley, B., Pearson, J. R. A., & Tooley, M. J. (1993). Stomatal density responds to the glacial cycle of environmental change. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, *251*(1331), 133-138. https://doi.org/10.1098/rspb.1993.0019

Bertolino, L. T., Caine, R. S., & Gray, J. E. (2019). Impact of stomatal density and morphology on water-use efficiency in a changing world. *Frontiers in Plant Science*, *10*, 225. https://doi.org/10.3389/fpls.2019.00225

Bhattacharjee, S. K. (1980). Native jasmine of India. *Indian Perfumes*, *24*(3), 126-133.

Chaitanya, H. S., Nataraja, S., & Krishnappa, M. (2020). Studies on morphological and genetic diversity of jasmine ecotypes of coastal Karnataka. *Ecology, Environment and Conservation*, *26*, S49-S55.

Clarke, I., & Lee, H. (2019). *Name that flowers: The identification of flowering plants* (3rd ed.). Melbourne University Press.

Coneva, V., & Chitwood, D. H. (2018). Genetic and developmental basis for increased leaf thickness in the *Arabidopsis* Cvi ecotype. *Frontiers in Plant Science*, *9*, 322. https://doi.org/10.3389/fpls.2018.00322

Demole, E., Lederer, E., & Mercier, D. (1962). Isolement et détermination de la structure du jasmonate de méthyle, constituant odorant caractéristique de l'essence de jasmin [Isolation and determination of the structure of methyl jasmonate, a characteristic odorous constituent of jasmine oil]. *Helvetica Chimica Acta*, *45*(2), 675-685. https://doi.org/10.1002/hlca.19620450233

Green, P. S., & Miller, D. M. (2009). *The genus* Jasminum *in cultivation*. Royal Botanic Gardens.

Hartati, S., & Darsana, L. (2015). Karakterisasi anggrek alam secara morfologi dalam rangka pelestarian plasma nutfah [Morphological characterization of natural orchids for germplasm conservation]. *Jurnal Agronomi Indonesia*, *43*(2), 133-139. https://doi.org/10.24831/jai.v43i2.10419

Haworth, M., Gallagher, A., Sum, E., Hill-Donnelly, M., Steinthorsdottir, M., & Mcelwain, J. (2014). On the reconstruction of plant photosynthetic and stress physiology across the Triassic-Jurassic boundary. *Turkish Journal of Earth Sciences*, *23*(3), 321-329. https://doi.org/10.3906/yer-1202-4

Haworth, M., Marino, G., Materassi, A., Raschi, A., Scutt, C. P., & Centritto, M. (2023). The functional significance of the stomatal size to density relationship: Interaction with atmospheric [$CO_2$] and role in plant physiological behaviour. *Science of The Total Environment*, *863*, 160908. https://doi.org/10.1016/j.scitotenv.2022.160908

Heyneke, E., Luschin-Ebengreuth, N., Krajcer, I., Wolkinger, V., Müller, M., & Zechmann, B. (2013). Dynamic compartment specific changes in glutathione and ascorbate levels in *Arabidopsis* plants exposed to different light intensities. *BMC Plant Biology*, *13*, 104. https://doi.org/10.1186/1471-2229-13-104

Hu, W., Lu, Z., Meng, F., Li, X., Cong, R., Ren, T., Sharkey, T. D., & Lu, J. (2020). The reduction in leaf area precedes that in photosynthesis under potassium deficiency: The importance of leaf anatomy. *New Phytologist*, *227*(6), 1749-1763. https://doi.org/10.1111/nph.16644

Jeyarani, J. N., Yohannan, R., Vijayavalli, D., Dwivedi, M. D., & Pandey, A. K. (2018). Phylogenetic analysis and evolution of morphological characters in the genus *Jasminum* L. (Oleaceae) in India. *Journal of Genetics*, *97*, 1225-1239. https://doi.org/10.1007/s12041-018-1019-4

Li, Q., & Kubota, C. (2009). Effects of supplemental light quality on growth and phytochemicals of baby leaf lettuce. *Environmental and Experimental Botany*, *67*(1), 59-64. https://doi.org/10.1016/j.envexpbot.2009.06.011

Marfirani, M., Rahayu, Y. S., & Ratnasari, E. (2014). Pengaruh pemberian berbagai konsentrasi filtra tumbi bawang merah dan rootnote-F terhadap pertumbuhan stek Melati "Rato Ebu" [Effect of various concentrations of onion filtrate and rootone-F on the "Rato Ebu" cuttings jasmine growth]. *LenteraBio*, *3*(1), 73-76.

Musalamah, Rianawati, S., & Rahardjo, B. (2019). Respons tiga genotipe melati terhadap pemupukan pada tanah Inseptisol [Responses of three jasmine genotypes to fertilization in Inceptisol fields]. In *Prosiding Seminar Nasional Varietasku untuk Negeri*: *Pemanfaatan Varietas Lokal untuk Menunjang Pertumbuhan Ekonomi Masyarakat* (pp. 61-71). Indonesian Agency for Agricultural Research and Development Press.

Nayak, B. S., & Mohan, K. (2007). Influence of ethanolic extract of *Jasminum grandflorum* Linn. flower on wound healing activity in rats. *Indian Journal of Physiology and Pharmacology*, *51*(2), 189-194.

Nguyen, T. P. D., Tran, T. T. H., & Nguyen, Q. T. (2019). Effects of light intensity on the growth, photosynthesis and leaf microstructure of hydroponic cultivated spinach (*Spinacia oleracea* L.) under a combination of red and blue LEDs in house. *International Journal of Agricultural Technology*, *15*(1), 75-90.

Palupi, T. I., Prasetyo, E., & Mukson, M. (2019). Analisis pendapatan bunga melati *Jasminum sambac* di Kabupaten Batang, Provinsi Jawa Tengah [Income analysis of jasmine flower *Jasminum sambac* in Batang Regency Central Java Province]. *Soca: Jurnal Sosial Ekonomi Pertanian*, *13*(3), 396-408.

Sari, D. P., & Harlita. (2018). Preparasi hands free section dengan teknik replika untuk identifikasi stomata [Hand free section preparation through replica technique for stomata identification]. *Proceeding Biology Education Conference*, *15*(1), 660-664.

Sari, M., & Tamami, N. D. B. (2020). Struktur, perilaku, dan kinerja usaha ronce melati Rato Ebhu di Desa Tunjung Kecamatan Burneh Kabupaten Bangkalan [The structure, behavior, and performance of Rato Ebhu's jasmine ronce business in Tunjung Village, Burneh District, Bangkalan Regency]. *Agriscience*, *1*(1), 292-307. https://doi.org/10.21107/agriscience.v1i1.8018

Shabala, S., Hariadi, Y., & Jacobsen, S.-E. (2013). Genotypic difference in salinity tolerance in quinoa is determined by differential control of xylem $Na^+$ loading and stomatal density. *Journal of Plant Physiology*, *170*(10), 906-914. https://doi.org/10.1016/j.jplph.2013.01.014

Shekhar, S., & Prasad, M. P. (2015). Evaluation of antimicrobial activity of *Jasminum* species using solvent extracts against clinical pathogens. *World Journal of Pharmacy and Pharmaceutical Sciences*, *4*(5), 1247-1256.

Tamami, N. D. B. (2012). Potensi usahatani melati Ratoh Ebuh sebagai komoditi unggulan daerah di Jawa Timur [The potential of jasmine Rattoh Ebuh farming as a regional leading commodity in East Java]. *Agriekonomika*, *1*(2), 160-180.

Yohanan, R., Jeyarani, N. J., Devipriya, V., Rather, S. A., Kasana, S., Thakur, J., Dwivedi, M. D., & Pandey, A. K. (2020). Evaluating genetic diversity within genus *Jasminum* L. (Oleaceae) using intersimple sequence repeats (ISSR) marker. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *90*, 531-540. https://doi.org/10.1007/s40011-019-01124-7

Pertanika J. Trop. Agric. Sci. 47 (4): 1087 - 1100 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Genetic Diversity of Indonesian Pineapple (*Ananas comosus* (L.) Merr.) Cultivars Based on ISSR Markers

**Risyda Hayati and Rina Sri Kasiamdari***

*Faculty of Biology, Universitas Gadjah Mada, Jl. Teknika Selatan, Sekip Utara, Yogyakarta 55281, Indonesia*

## ABSTRACT

Pineapple (*Ananas comosus* (L.) Merr.) is the third-most important tropical fruit traded and widely cultivated in Indonesia with various cultivars. This study identifies genetic diversity and determines the phenetic relationship of nine pineapple accessions based on inter-simple sequence repeat (ISSR) markers. Four ISSR primers were utilized for genetic diversity analysis and diversity relationships using POPGENE 1.31 and MVSP 3.2 to form a dendrogram. The results showed that nine pineapple accessions revealed the successful amplification of 50 DNA bands, 46 polymorphic, with a percentage average of 89.38%. The calculation result of the effective alleles (Ne), Nei's gene diversity (h), Shannon information index (I), and polymorphism information content (PIC) showed that ISSR 1 had the highest value and ISSR 16 had the lowest value. The average of the Ne value was 1.44; the average of h was 0.28, which indicated low genetic variation; the average of (I) was 0.43, which indicated that not all groups had the same frequency; the averages of the PIC value of 0.28 which showed that the four ISSR primers used were somewhat informative. The results of the phenetic relationship based on ISSR molecular markers showed two clusters that separated the accession of 'Spanish' from the accession of 'Queen' and 'Cayenne.' This resulting study showed that ISSR analysis was suitable for studying genetic diversity among pineapple cultivars.

*Keywords*: *Ananas comosus*, cultivar, Indonesia, ISSR, phenetic relationship

ARTICLE INFO

*Article history:*
Received: 23 October 2023
Accepted: 20 December 2023
Published: 27 September 2024

DOI: https://doi.org/10.47836/pjtas.47.4.02

*E-mail addresses*:
risydahayati@mail.ugm.ac.id (Risyda Hayati)
rkasiamdari@ugm.ac.id (Rina Sri Kasiamdari)
* Corresponding author

## INTRODUCTION

The pineapple (*Ananas comosus* (L.) Merr.) is a plant belonging to the Bromeliaceae family and produces significant fruit for the economy. It was in agreement with the statements made by Hassan et al. (2011) and Wang et al. (2017), who stated that

ISSN: 1511-3701
e-ISSN: 2231-8542

after bananas and oranges, pineapple was the third-most important tropical fruit traded. As a tropical nation with a variety of agroclimates, Indonesia can support the growth of pineapple production and is currently the largest pineapple-producing nation in the world (Budianingsih et al., 2017; Statista, 2024). A total of 336,102 and 261,769 tons of pineapples were produced in Central Java and Riau Provinces, respectively (Badan Pusat Statistik Provinsi Jawa Tengah, 2022; Badan Pusat Statistik Provinsi Riau, 2023).

Pineapple cultivars have been divided into groups based on differences in morphological characteristics, especially in their leaves and fruits (Wang et al., 2017). Pineapple cultivar groups that have been cultivated in Indonesia were 'Queen', 'Cayenne', 'Abacaxi', and 'Spanish' (Amda et al., 2020; Hernosa et al., 2022; Rosmaina et al., 2021). Analysis of morphological characteristics has become a classic approach to distinguishing plant cultivars, so this character became the main character used in the classification and initial identification of taxa, both phenotypic and genotypic (Martiwi et al., 2020; Valencia & Alcasid, 2014). However, this character has a weakness, which can be plasticity, because environmental factors influenced it, so other characters are needed for support and comparison (Singh, 2010).

Molecular characters are one of the supporting and comparative characters that can be used in the classification and identification of taxa. This character is stable, can be detected in all plant tissues, is not influenced by environmental factors, and can be detected in all phases of growth (Pabendon et al., 2007; Zulfahmi, 2013). Due to their speed, accuracy, and strong results, molecular marker techniques currently appear to be the method of choice for a variety of applications, displaying excellent reliability to supplement and enhance conventional approaches (Ismail et al., 2020). Furthermore, molecular markers are now frequently employed as a tool to assess the accuracy of plant taxonomy classifications. Through genetic characterization and fingerprinting, genetic analysis, relationship mapping, and molecular breeding, molecular marker technology is also effective in improving crops (Valencia & Alcasid, 2014).

DNA molecular techniques were used to investigate plant genetic variability to detect and establish relationships at the cultivar and species levels (Souza et al., 2017). One of the molecular markers frequently used to analyze genetic diversity and identify phenetic relationships was inter-simple sequence repeat (ISSR). ISSR is a polymerase chain reaction (PCR)-based genetic marker that has been developed as an anonymous and one of the markers with repeated sequences in the form of DNA fragments with a size of 100-3,000 bp located between microsatellite regions (Mohamed et al., 2014; Napitu et al., 2016). ISSR is also a molecular marker with a number of benefits, including being simple, quick, and affordable like the random amplified polymorphic DNA (RAPD) molecular marker, not requiring sequence data during primary construction, requiring

only PCR for analysis, and randomly distributing the process across the genome (Godwin et al., 1997; Vijayan, 2005; Semagn et al., 2006). ISSR is a molecular marker frequently employed to examine plant genetic variability, and it is more reproducible than RAPD since it can be used in most situations and has been very effective in genetic fingerprinting and diversity analysis (Arif et al., 2020; Godwin et al., 1997).

Previous studies have effectively used ISSR molecular techniques to analyze genetic variation in pineapple cultivars. In a study by Wang et al. (2017) on the genetics of pineapple accessions from 10 pineapple-producing countries, 13 ISSR primers amplified 96 bands, 91 of which were polymorphic. A total of 56 DNA bands were amplified in the study of Vanijajiva (2012) about the assessment of genetic diversity and relationships in pineapple cultivars from Thailand using ISSR markers, of which 27 were polymorphic. A study by Harahap et al. (2022) about genetic similarities in plantlet pineapples in vitro from North Sumatra using ISSR molecular markers reported that out of the six primers from the 192 amplified bands, 23 bands of polymorphic loci were obtained. This study identifies genetic diversity and determines phenetic relationships of pineapple accessions from Central Java and Riau Provinces based on molecular characters using ISSR markers.

## MATERIALS AND METHODS

### Sampling Collection

Five districts were chosen for sample collection: Wonosobo, Magelang, and Pemalang Regencies in Central Java Province: Kampar and Siak Regencies in Riau Province (Figure 1). The samples collected were pineapple leaves from farmers' plantations with the criteria for pineapple plants to be sampled 1–3 years old and flowering. The leaves were placed in ziplock plastic, labeled, and stored at -20°C in the freezer. Nine pineapple

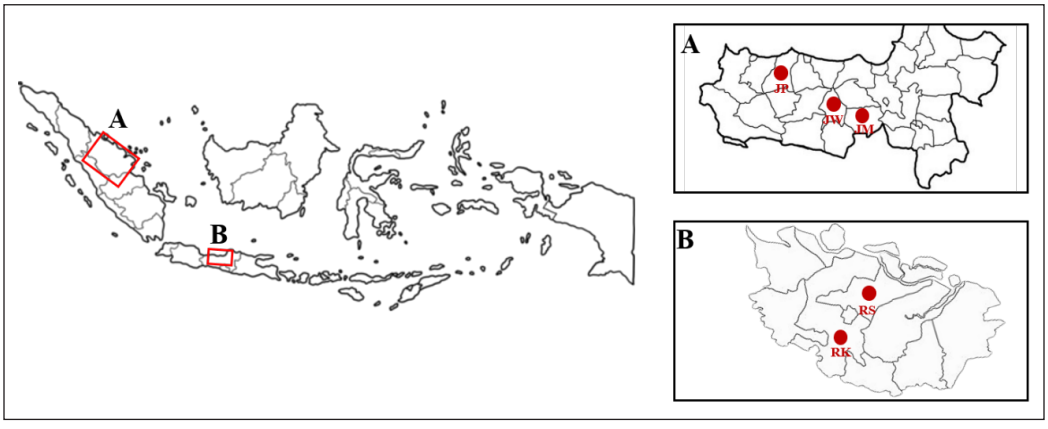



*Figure 1*. Five pineapple sampling locations in Indonesia: (A) Central Java Province (JW = Wonosobo: 7°22'12.7"S 109°52'32.4"E, JM = Magelang: 7°35'46.8"S 110°10'55.5"E, and JP = Pemalang: 7°09'36.9"S 109°18'39.3"E); (B) Riau Province (RK = Kampar: 0°25'15.7"N 101°14'44.8"E and RS = Siak: 1°05'12.4"N 102°07'52.5"E)
*Note*. S = South; W = West; E = East; N = North

Table 1
*Collection of pineapple samples from Central Java and Riau Provinces*

| Cultivar | Local name | Accession code | Accession location | Province |
|---|---|---|---|---|
| 'Cayenne' | Kopyor Pineapple | JW01 | Duren Sawit, Wonosobo | Central Java |
| 'Cayenne' | Kopyor Pineapple | JW02 | Duren Sawit, Wonosobo | |
| 'Cayenne' | Benggolo Pineapple | JM01 | Kembang Limus, Magelang | |
| 'Cayenne' | Benggolo Pineapple | JM02 | Kembang Limus, Magelang | |
| 'Queen' | Madu Pineapple | JP01 | Beluk, Pemalang | |
| 'Queen' | Kualu Pineapple | RK01 | Kualu, Kampar | Riau |
| 'Queen' | Moris Pineapple | RK02 | Pagaruyung, Kampar | |
| 'Spanish' | Madu Pineapple | RK03 | Pagaruyung, Kampar | |
| 'Queen' | Moris Pineapple | RS01 | Sungai Apit, Siak | |

accessions from Central Java and Riau Provinces were analyzed molecularly at the Genetic Engineering Laboratory, Center for Biotechnology Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia (Table 1).

**Data Collection and Analysis**

A total of 0.1 g of leaf samples were used for DNA isolation using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1987) with modifications. The liquid nitrogen was used to compress the leaf samples until smooth and then transferred to a 2 ml microtube with 1,400 µl of CTAB buffer (HiMedia, USA). The samples were incubated in a 60$^{o}$C water bath for 60 min, switching positions every 10 min to maintain homogeneity. After cooling, add 500 µl of chloroform: isoamyl alcohol 24:1 (CIAA, Merck, Germany), and then vortex for 2 min. The sample was then centrifuged for 15 min at 8,050 x *g*. The supernatant was poured into a microtube, 500 µl of CIAA was added, and the microtube was recentrifuged at 8,050 x *g* for 15 min. Removing the second centrifuge supernatant and transferring it to a new 1.5 µl microtube. Then, the supernatant was added with sodium acetate as much as 1/10 of the volume of the supernatant obtained and 2/3 of the total volume. The microtubes were gently turned over so that all solutions were homogeneous and stored in a -20$^{o}$C freezer for 24 hr. The sample was recently centrifuged for 10 min at 8,050 x *g*. Supernatants were removed, and pellets in DNA deposits were washed using 70% ethanol solution (Merck, Germany). Ethanol was removed from the microtubes, and DNA pellets were dried in the air. In the last stage, 50 µl of Tris-EDTA (TE) buffer (HiMedia, USA) was added to the microtube and stored in the freezer at -20$^{o}$C. Nanodrop (MaestroGen, Taiwan) was used to test DNA concentration and purity at 260/280 nm absorbance.

Four ISSR sequence primers were used for DNA amplification, as reported by Wang et al. (2017) (Table 2) with the ISSR-PCR method. A total of 25 µl of PCR mix, including 2 µl of DNA template (25 ng), 2 µl of ISSR primer (10 pmol), 12.5 µl

Table 2
*Sequences of inter-simple sequence repeat (ISSR) primer used in DNA amplification (Wang et al., 2017)*

| Primer | Primer sequences (5' – 3') |
|---|---|
| ISSR 1 | 5' CACACACACACACACAGT3' |
| ISSR 3 | 5' GAGAGAGAGAGAGAGA(CT)C(AG)3' |
| ISSR 16 | 5'GTGTGTGTGTGTGTGTC3' |
| ISSR 24 | 5'GACAGACAGACAGACA3' |

of PCR Kit ready mix MyTaqTM HS Red Mix (Bioline, United Kingdom), and 8.5 µl of nuclease-free water, were used for the PCR reaction.

The following PCR conditions are used to carry out ISSR amplification: initial denaturation at 94°C for 5 min, followed by denaturation with 40 cycles for 30 s at 94°C, annealing at 50–51°C for 45 s, continued with the final extension at 72°C for 90 s. The electrophoresis of DNA bands on 1.8% agarose gel in 50 ml of Tris-borate-EDTA (TBE) 1× buffer (HiMedia, USA) and using florosafe DNA dye (Boca Scientific Inc., USA) as much as 4 µl revealed to determine the results of the DNA amplification. Electrophoresis was performed at 110 V for 30 min and then visualized using an ultraviolet (UV) transilluminator.

The molecular data in the form of DNA bands that appear are given a score with the provisions of 0 (invisible DNA band) and 1 (visible DNA band) so that the data obtained is binary. The number of polymorphic bands (NPB), percentage of polymorphic bands (PPB), average number of Ne, average h, and average I were all calculated for each primer using the POPGENE 1.31 program (Yeh et al., 1999). The following formula was also used to compute the PIC (De Riek et al., 2001):

$$PIC = 1 - [f^2 + (1 - f)^2]$$

where, 'f' indicates the marker's frequency within the data set. DNA band scoring results were used to calculate the similarity matrix of pineapple accessions using the simple matching coefficient (SMC) formula. To create a dendrogram, a cluster analysis utilizing unweighted pair group methods with arithmetic averages (UPGMA) and multi variate statistical package (MVSP) software version 3.2 was used.

## RESULTS

A general morphological view can distinguish three pineapple cultivars from the nine accessions observed, one of which can be seen in the morphological characters of the fruit, such as the color of the fruit when ripe, fruit shape, and crown shape (Figure 2).

Nine pineapple accessions from Central Java and Riau Provinces, Indonesia, were tested by DNA amplification using four ISSR primers (ISSR 1, ISSR 3, ISSR 16, and ISSR 24), and the result revealed the successful amplification of 50 DNA bands, 46 of which were polymorphic. Each primer produced 8 to 20 DNA bands that were amplified (Table 3 and Figure 3). ISSR 24 produced the highest number of DNA bands (20 bands), whereas ISSR 3 and 16 produced

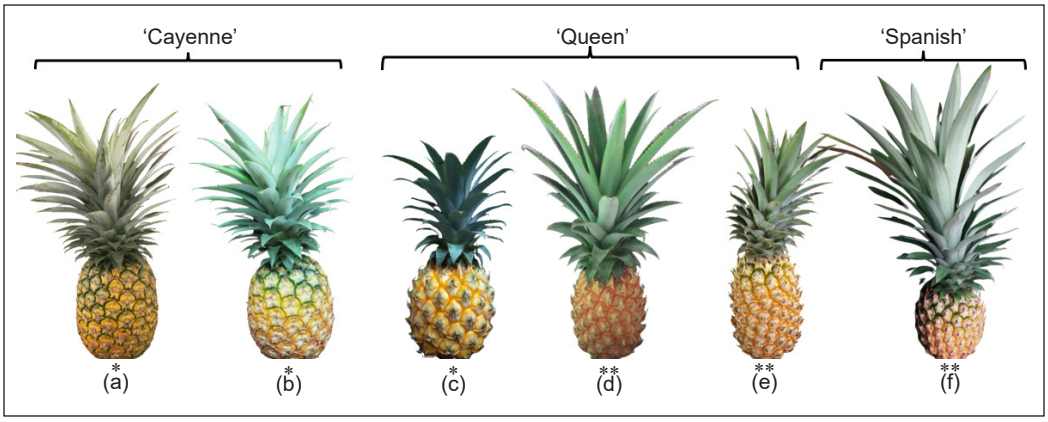



*Figure 2*. Morphological characters of fruit in three Indonesian pineapple cultivars: (a-b) 'Cayenne', (c-e), 'Queen', and (f) 'Spanish'
*Note*. * = Central Java Province; ** = Riau Province

the lowest number of DNA bands (8 bands) (Table 3 and Figure 3). However, the primer that showed the highest percentage of polymorphism in this study was in ISSR 1 (100%) and the lowest in ISSR 16 (75%) (Table 3 and Figure 3). The size of the amplified DNA bands in this study ranged from 175–2,000 bp (Table 3 and Figure 3). The analysis indicated that the average percentage of polymorphic DNA (PPB) in four ISSR primers was 89.38%.

The average of Ne, average h, average I, and average PIC values were calculated (Table 3), reported that between ISSR 1, ISSR 3, ISSR 16, and ISSR 24 showed that ISSR 1 had the highest value and ISSR 16 had the lowest value. The average value of Ne was 1.44. At the same time, the low genetic variety was suggested by the average value of h being 0.28, and not all groups had the same frequency, as shown by the average value of I being 0.43. The four ISSR primers used in this study were found to have a PIC value ranging from 0.19 to 0.33, with an average of 0.28, indicating that they were considered somewhat informative.

Table 3
*Diversity information parameters on the four inter-simple sequence repeat (ISSR) markers*

| Primers | TNB | NPB | PPB (%) | Ne | h | I | PIC |
|---|---|---|---|---|---|---|---|
| ISSR 1 | 14 | 14 | 100 | 1.52 | 0.33 | 0.50 | 0.33 |
| ISSR 3 | 8 | 7 | 87.5 | 1.46 | 0.28 | 0.44 | 0.28 |
| ISSR 16 | 8 | 6 | 75 | 1.28 | 0.19 | 0.30 | 0.19 |
| ISSR 24 | 20 | 19 | 95 | 1.49 | 0.30 | 0.46 | 0.30 |
| Total | 50 | 46 | 357.50 | 5.75 | 1.10 | 1.70 | 1.10 |
| Average | 12.50 | 11.50 | 89.38 | 1.44 | 0.28 | 0.43 | 0.28 |

*Note*. TNB = Total number of bands; NPB = Number of polymorphic bands; PPB = Percentage polymorphic bands; Ne = Average of effective alleles; h = Average Nei's gene diversity; I = Average Shannon information index; PIC = Polymorphism information content

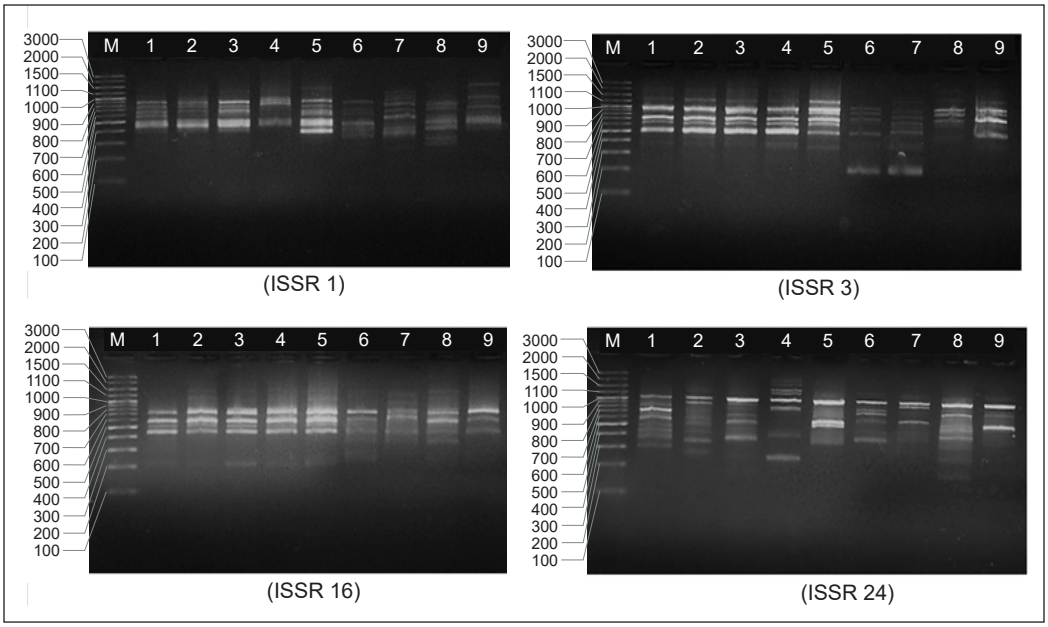



*Figure 3*. The inter-simple sequence repeat (ISSR) profiles of ISSR1, ISSR 3, ISSR 16, and ISSR 24 on *Ananas comosus*

*Note*. M = Marker 100 bp; Lane 1 = 'Cayenne' (JW01); Lane 2 = 'Cayenne' (JW02); Lane 3 = 'Cayenne' (JM01); Lane 4 = 'Cayenne' (JM02); Lane 5 = 'Queen' (JP01); Lane 6 = 'Queen' (RK01); Lane 7 = 'Queen' (RK02); Lane 8 = 'Spanish' (RK03); Lane 9 = 'Queen' (RS01)

The similarity value in nine pineapple accessions based on the dendrogram (Figure 4) ranged from 59–86%. Based on cluster analysis using ISSR markers on nine pineapple accessions, there were two primary clusters, namely clusters A and B, at the phenon line of 60% similarity index (Figure 4). Cluster A was an accession from 'Spanish,' while Cluster B was from 'Queen' and 'Cayenne'. Cluster A consisted of one accession from 'Spanish' (RK03), while cluster B consisted of four accessions from 'Queen' (RK01, RK02, RS01, JP01) and four accessions from 'Cayenne' (JW01, JW02, JM01, JM02).

Principal component analysis (PCA) based on 50 bands ISSR of nine accessions pineapple showed two principal components (PC1 and PC2) (data not shown). The UPGMA clustering (Figure 4) and the distribution pattern from PCA (Figure 5), respectively, were associated. The first principal component (PC1) explained 23.54% of the variation. In comparison, the second principal component (PC2) explained 21.83% of the variation, and the total value of the cumulative variation from PC1 and PC2 was 45.37% (data not shown).

## DISCUSSION

This study was the first report on ISSR markers being applied to identify genetic differences in pineapple accessions from Central Java and Riau Provinces. Various pineapple accessions from Riau Province

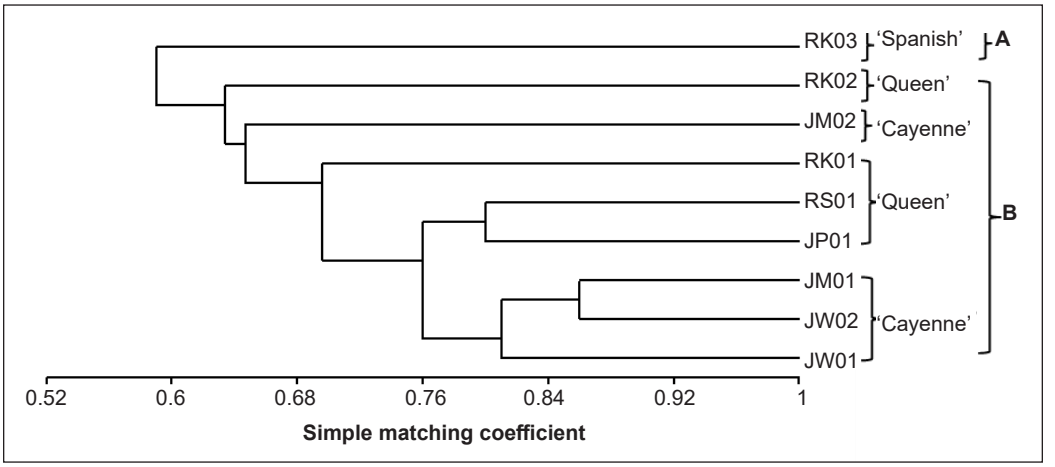



*Figure 4*. Dendrogram of nine pineapple accessions based on inter-simple sequence repeat (ISSR) markers

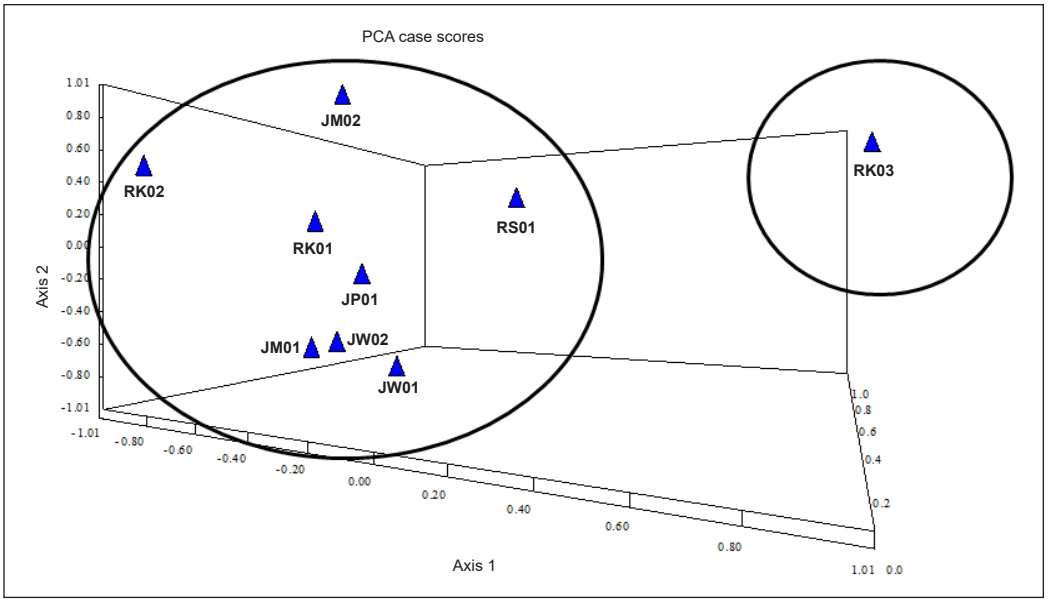



*Figure 5*. Three-dimensional plot of principal component analysis of nine accessions pineapple based on inter-simple sequence repeat markers

*Note*. PCA = Principal component analysis; JW01 = 'Cayenne' (Wonosobo); JW02 = Cayenne' (Wonosobo); JM01 = 'Cayenne' (Magelang); JM02 = 'Cayenne' (Magelang); JP01 = 'Queen' (Pemalang); RK01 = 'Queen' (Kampar); RK02 = 'Queen' (Kampar); RK03 = 'Spanish' (Kampar); RS01 = 'Queen' (Siak)

showed a substantial amount of genetic variety. In previous studies, the genetic diversity of pineapple accessions from Riau Province was conducted by RAPD profiles, and the result showed a high polymorphic percentage value of 53–91% (Rosmaina et al., 2022).

The results of this study used four ISSR primers that were previously used by Wang et al. (2017) for pineapple diversity

in ten pineapple-producing countries. The number of DNA bands in the four ISSR primers amplified in the result of this study had a greater value (50 bands) than the result of the study by Wang et al. (2017) (44 bands), with a range from 8–20 and 8–14, respectively. Moreover, the highest number of DNA bands from four ISSR primers was ISSR 24, totaling 20 bands. The result was in agreement with Wang et al. (2017) study results, which showed that the ISSR 24 produced the most DNA bands. It showed that the same primer (ISSR 24) in different studies can produce a high number of bands in pineapple identification. However, the percentage of polymorphic bands showed different results, that were in this study showed a value of 100% only found in ISSR 1, while the study by Wang et al. (2017) used the same four primers and showed that all primers produced percentage polymorphic band value of 100%. It showed that the total percentage value of polymorphic DNA from Wang et al. (2017) was higher than our result.

Each primer produced a varied number of DNA bands and polymorphic bands. It explained that the ISSR molecular marker was a powerful tool for genetic studies of pineapples because it could identify very low levels of genetic variation. Rakoczy-Trojanowska and Bolibok (2004) and Reddy et al. (2002) stated that ISSR primers were molecular markers that can produce high polymorphisms. According to Vanijajiva (2012), a high polymorphism level suggested that the probability of mutation in pineapple DNA is considerable. The extensive geographic distribution and significant ecological variables may be responsible for the high mutation probability. It was also supported by Aradhya et al. (1994), who suggested that ecological isolation may cause morphological differences among the species of *Ananas*. It might also suggest that finding polymorphisms at the species level is a high priority for random primers. Moreover, Gholami et al. (2021) stated that ISSR markers proved useful for distinguishing and identifying the relationships among species and populations collected from geographically different locations.

Although the ISSR molecular marker has several advantages over other molecular markers, it was rarely used to identify the genetic diversity of pineapples in Indonesia. It was also supported by Wang et al. (2017), who stated that ISSR molecular markers were still rarely used in analyzing the genetic diversity of pineapple. The study related to pineapple genetic diversity in Indonesia that used ISSR molecular markers was Harahap et al. (2021, 2022) about the genetic variability of pineapple plantlets and the genetic similarity of pineapples cultivated in vitro from Sipahutar, North Sumatra, reported that a total of six and ten ISSR primers used in both Harahap studies, respectively, the results revealed genetic similarity of plantlets and in vitro culture pineapple had similarity level of 76–97% and 75–94%. This study resulted in a higher percentage of polymorphism, 75–100% from four ISSR primers.

The Ne, I, h, and PIC calculations (Table 3) showed that ISSR 1 had the highest value in each parameter. This result was different

from Wang et al. (2017), which indicated that ISSR 24 had the highest value on each of those parameters. The average Ne calculation represented the number of Ne derived from each population. It was the homozygosity's reciprocal or inverse value. The higher the value of Ne, the more the individuals are heterozygous (Solin et al., 2014). The h value was utilized to measure genetic diversity and genetic divergence and analyze population relationships. The calculation of gene frequencies at each locus produces heterozygosity. (Terryana et al., 2020). According to Nei (1978), if the heterozygosity (h) value ranges from 0.1–0.4, it indicates low genetic variation; if it ranges from 0.5–0.7, it indicates moderate genetic variation; and if it ranges from 0.8–1.0, it indicates high genetic variation. The heterozygosity (h) value ranges from 0 (zero) to 1 (one). The I value was the most suitable estimator to describe variation at multiallelic loci such as microsatellites (Konopiński, 2020). Then, according to Ramezani (2012), the Shannon diversity index is normalized to have a value between 0 and 1: lower values represent greater diversity, while higher values represent less diversity. A value of 1 for the index indicates that all groups experience the same frequency. PIC is a parameter gauges how well it can spot polymorphism among a population's members. The better this ability, the higher the parameter's value. It provides one of genetic research's markers' quality indicators (Serrote et al., 2020). The four ISSR primers used in this study were shown to have an average PIC value of 0.28, indicating that they are thought to be somewhat informative. In contrast, a study by Wang et al. (2017) revealed an average PIC value of 0.22, indicating that they are seen to be not very informative. According to a study by Serrote et al. (2020), PIC values greater than 0.5 are regarded to be very informative for primers, while values between 0.25 and 0.50 are regarded to be somewhat informative, and values less than 0.25 are regarded to be not very informative. According to Kaki et al. (2020), the high PIC suggested rare alleles and high polymorphism in a single gene locus, which can be utilized to distinguish genotypes.

The result of cluster analysis (Figure 4) and PCA (Figure 5) using ISSR markers on nine pineapple accessions showed that the accession of 'Spanish' (Cluster A) separated from the accession of 'Queen' and 'Cayenne' (Cluster B). This result was supported by Hadiati et al. (2018), Rattanathawornkiti et al. (2016), and Rosmaina et al. (2022), which reported that 'Queen' and 'Cayenne' grouped into one cluster based on molecular characters. Using different ISSR primer types, similar results were obtained where 'Queen' and 'Cayenne' were grouped in one cluster and separated with 'Spanish.' Genetic variations can be influenced by a number of factors, including the quality and intensity of DNA electrophoresis bands, according to Harahap et al. (2022). The purity and concentration of the DNA separated, and the location of the main attachment

sites can impact the strength of DNA bands between samples. Furthermore, it was impacted by the competition for primer attachment sites on the isolated DNA, resulting in one band amplifying in multiplex while others are not amplified (Bilodeau et al., 2012). The similarity value in nine pineapple accessions based on the dendrogram (Figure 2) ranged from 59–86%. According to Jannah et al. (2022), populations with low genetic similarity showed how isolation increases genetic variety, which leads to a certain characteristic's emergence. Moreover, Poerba et al. (2019) added that the isolation drove the evolution of a particular trait with similar genetic properties, as opposed to the distinct environment or geographic condition that gave rise to other adaptation patterns and genetic characteristics.

## CONCLUSION

Nine pineapple accessions used to study the genetic diversity showed the average Ne value of 1.44 and h value of 0.28, indicating low genetic variation and a percentage value of polymorphic bands in the four ISSR (ISSR 1, ISSR 3, ISSR 16, ISSR 24) primers of 89.38%. The I value was 0.43, indicating that not all groups had the same frequency, and the PIC average was 0.28, indicating that the four ISSR primers employed were thought to be somewhat informative. The results of the phenetic relationship based on ISSR molecular markers showed two clusters that separated the accession of 'Spanish' from the accession of 'Queen' and 'Cayenne.'

## ACKNOWLEDGEMENTS

This research is supported by the Final Project Recognition Grant Universitas Gadjah Mada Number 5075/UN1.P.II/Dit-Lit/PT.01.01/2023. It is part of Risyda Hayati's thesis and is under the supervision of Rina Sri Kasiamdari.

## REFERENCES

Amda, P. P. E., Hanfiah, D. S., & Kadhinata, E. H. (2020). Karakterisasi morfologis dan hubungan kekerabatan tanaman nanas (*Ananas comosus* (L.) Merr.) di Kabupaten Kampar dan Siak Provinsi Riau [Morphological characterization and relationships of pineapple (*Ananas comosus* (L.) Merr.) in Kampar and Siak Districts, Riau Province]. *Rhizobia*, *2*(2), 134–144. https://doi.org/10.36985/rhizobia.v9i2.313

Aradhya, M. K., Zee, F., & Manshardt, R. M. (1994). Isozyme variation in cultivated and wild pineapple. *Euphytica*, *79*, 87–99. https://doi.org/10.1007/BF00023580

Arif, M. F., Subositi, D., Sari, A. N., Aristya, G. R., Lesmana, I., & Kasiamdari, R. S. (2020). Genetic Diversity of green chireta (*Andrographis paniculata* (Burm.f.) Wall. Ex Nees.) from Indonesia based on ISSR and RAPD markers. *Malaysian Applied Biology*, *49*(1), 61–68. https://doi.org/10.55230/mabjournal.v49i1.1655

Bilodeau, G. J., Koike, S. T., Uribe, P., & Martin, F. N. (2012). Development of an assay for rapid detection and quantification of *Verticillium dahliae* in soil. *Phytopathology*, *102*(3), 331–343. https://doi.org/10.1094/PHYTO-05-11-0130

Badan Pusat Statistik Provinsi Jawa Tengah. (2022). *Produksi buah-buahan dan sayuran tahunan menurut jenis tanaman di Provinsi Jawa Tengah, 2020-2021* [Annual fruit and vegetable production by plant type in Central Java

Province, 2020-2021]. https://jateng.bps.go.id/statictable/2022/03/15/2540/produksi-buah-buahan-menurut-kabupaten-kota-dan-jenis-tanaman-di-provinsi-jawa-tengah-2020---2021.html

Badan Pusat Statistik Provinsi Riau. (2023). *Statistika tanaman sayuran dan buah-buahan Provinsi Riau* [Statistics of vegetable and fruit crops in Riau Province]. https://riau.bps.go.id/publication/2023/09/19/6e9fe2a414c109c867b98727/statistik-tanaman-sayuran-dan-buah-buahan-provinsi-riau-2022.html

Budianingsih, L., Hadi, S., & Edwina, S. (2017). Agribisnis nenas di Kecamatan Tambang Kabupaten Kampar [Pineapple agribusiness in Tambang District, Kampar Regency]. *Jurnal Online Mahasiswa Fakultas Pertanian Universitas Riau*, *4*(1), 1–11.

De Riek, J., Calsyn, E., Everaert, I., Van Bockstaele, E., & De Loose, M. (2001). AFLP based alternatives for the assessment of distinctness, uniformity and stability of sugar beet varieties. *Theoretical and Applied Genetics*, *103*, 1254–1265. https://doi.org/10.1007/s001220100710

Doyle, J. J., & Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, *19*(1), 11–15.

Gholami, S., Vafaee, Y., Nazari, F., & Ghorbani, A. (2021). Molecular characterization of endangered iranian terrestrial orchids using ISSR markers and association with floral and tuber-related phenotypic traits. *Physiology and Molecular Biology of Plants*, *27*, 53–68. https://doi.org/10.1007/s12298-020-00920-0

Godwin, I. D., Aitken, E. A. B., & Smith, L. W. (1997). Application of inter simple sequence repeat (ISSR) markers to plant genetics. *Electrophoresis*, *18*(9), 1524–1528. https://doi.org/10.1002/elps.1150180906

Hadiati, S., Prihatini, R., & Mansyah, E. (2018). Identifikasi molekuler dan analisis kekerabatan aksesi nenas menggunakan marka RAPD menunjang perakitan varietas unggul baru [Molecular identification and relationships among several pineapple accessions using RAPD marker to support the assembling new varieties]. *Jurnal Hortikultura*, *28*(1), 1–12. https://doi.org/10.21082/jhort.v28n1.2018.p1-12

Harahap, F., Afiva, A., Jannah, M., & Prasetya, E. (2021). ISSR based analysis of genetic variability of plantlets culture of pineapple (*Ananas comosus* L.) from Sipahutar, North Sumatera, Indonesia. *Biogenesis: Jurnal Ilmiah Biologi*, *9*(1), 35–41. https://doi.org/10.24252/bio.v9i1.17068

Harahap, F., Nusyirwan, N., Afiva, A., Prasetya, E., Suriani, C., Hasibuan, R. F. M., & Poerwanto, R. (2022). Short communication: Genetic similarity analysis of *in vitro* cultivated pineapple (*Ananas comosus*) from Sipahutar, North Sumatra, Indonesia using ISSR markers. *Biodiversitas*, *23*(11), 5623–5628. https://doi.org/10.13057/biodiv/d231111

Hassan, A., Othman, Z., & Siriphanich, J. (2011). Pineapple (*Ananas comosus* L. Merr.). In E. M. Yahia (Ed.), *Postharvest biology and technology of tropical and subtropical fruits: Mangosteen to white sapote* (pp. 194-218e). Woodhead Publishing. https://doi.org/10.1533/9780857092618.194

Hernosa, S. P., Siregar, L. A. M., Hanum, C., & Supriana, T. (2022). Morphological characterization and its relationship with preference for pineapple in Labuhan Batu Regency, Indonesia. *Asian Journal of Plant Sciences*, *21*(3), 379–388. https://doi.org/10.3923/ajps.2022.379.388

Ismail, S. N., Ghani, N. S. A., Razak, S. F. A., Abidin, R. A. Z., Yusof, M. F. M., Zubir, M. N., & Zainol, R. (2020). Genetic diversity of pineapple (*Ananas comosus*) germplasm in Malaysia using simple sequence repeat (SSR) markers. *Tropical Life Science Research*, *31*(3), 15–27. https://doi.org/10.21315%2Ftlsr2020.31.3.2

Jannah, N., Pharmawati, M., & Uslan. (2022). Genetic diversity of *Sterculia quadrifida* from Kupang based on ISSR profiles, stomatal density, and chlorophyll content. *Biodiversitas*, *23*(5), 2690–2698. https://doi.org/10.13057/biodiv/d230553

Kaki, A., Vafaee, Y., & Khadivi, A. (2020). Genetic variation of *Anacamptis coriophora*, *Dactylorhiza umbrosa*, *Himantoglossum affine*, *Orchis mascula*, and *Ophrys schulzei* in the western parts of Iran. *Industrial Crops and Products*, *156*, 112854. https://doi.org/10.1016/j.indcrop.2020.112854

Konopiński, M. K. (2020). Shannon diversity index: A call to replace the original Shannon's formula with unbiased estimator in the population genetics studies. *PeerJ*, *8*, e9391. https://doi.org/10.7717/peerj.9391

Martiwi, I. N. A., Nugroho, L. H., Daryono, B. S., & Susandarini, R. (2020). Morphological variability and taxonomic relationship of *Sorghum bicolor* (L.) Moench accessions based on qualitative characters. *Annual Research and Review in Biology*, *35*(6), 40–52. https://doi.org/10.9734/arrb/2020/v35i630234

Mohamed, E.-A. A. M., Dessoky, E. S., Attia, A. O., & Hassan, M. M. (2014). Evaluation of genetic fidelity of *in vitro* raised plants of the important medicinal plantharmal (*Rhazya Stricta* Decne) using RAPD and ISSR markers. *International Journal of Agricultural Science and Research*, *4*(3), 115–124.

Napitu, C. S. P. L. S., Chikmawati, T., & Djuita, N. R. (2016). Keberagaman genetik kerabat rambutan liar (*Nephelium* spp.) di Kabupaten Sanggau, Kalimantan Barat berdasarkan marka SSR dan ISSR [Genetic diversity of wild rambutan relatives (*Nephelium* spp.) in Sanggau Regency, West Kalimantan based on SSR and ISSR markers]. *Floribunda*, *5*(4), 115–125. https://doi.org/10.32556/floribunda.v5i4.2016.12

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, *89*(3), 583–590. https://doi.org/10.1093/genetics/89.3.583

Pabendon, M. B., Azrai, M., Kasim, F., & Mejaya, M. J. (2007). *Prospek penggunaan marka molekuler dalam program pemuliaan jagung* [Prospects for the use of molecular markers in maize breeding programs]. Balitsereal.

Poerba, Y. S., Martanti, D., & Ahmad, F. (2019). Genetic variation of wild *Musa acuminata* Colla from Indonesia. *BIOTROPIA*, *26*(2), 115–126. https://doi.org/10.11598/btb.2019.26.2.896

Rakoczy-Trojanowska, M., & Bolibok, H. (2004). Characteristics and a comparison of three classes of microsatellite-based markers and their application in plants. *Cellular and Molecular Biology Letters*, *9*(2), 221–238.

Ramezani, H. (2012). A Note on the normalized definition of Shannon's diversity index in landscape pattern analysis. *Environment and Natural Resources Research*, *2*(4), 54–60. https://doi.org/10.5539/enrr.v2n4p54

Rattanathawornkiti, K., Kanchanaketu, T., Suwanagul, A., & Hongtrakul, V. (2016). Genetic relationship assessment of pineapple germplasm in Thailand revealed by AFLP markers. *Genomics and Genetics*, *9*(2 & 3), 56–65. https://doi.org/10.14456/gag.2016.8

Reddy, M. P., Sarla, N., & Siddiq, E. A. (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. *Euphytica*, *128*, 9–17. https://doi.org/10.1023/A:1020691618797

Rosmaina., Elfianis, R., Almaksur, A., & Zulfahmi. (2021). Minimal number of morphoagronomic characters required for the identification of pineapple (*Ananas comosus*) cultivars in peatlands of Riau, Indonesia. *Biodiversitas*, *22*(9), 3854–3862. https://doi.org/10.13057/biodiv/d220931

Rosmaina., Febriandi, N., Ariyanti, E., Elfianis, R., Nilahayati., & Zulfahmi. (2022). Pineapple genetic diversity in Riau peat land assessed by random amplified polymorphic DNA (RAPD) marker. In *IOP Conference Series: Earth and Environmental Science* (Vol. 1114, No. 1, p. 012020). IOP Publishing. https://doi.org/10.1088/1755-1315/1114/1/012020

Semagn, K., Bjørnstad, Å., & Ndjiondjop, M. N. (2006). An overview of molecular marker methods for plants. *African Journal of Biotechnology*, *5*(25), 2540–2568.

Serrote, C. M. L., Reiniger, L. R. S., Silva, K. B., dos Santos Rabaiolli, S. M., & Stefanel, C. M. (2020). Determining the polymorphism information content of a molecular marker. *Gene*, *726*, 144175. https://doi.org/10.1016/j.gene.2019.144175

Singh, G. (Ed.) (2010). *Plant Systematics: An intregated approach* (3rd ed). CRC Press. https://doi.org/10.1201/b10255

Solin, N. W. N. M., Sobir., & Toruan-Mathius, N. (2014). Genetic diversity of DxP population yield component in oil palm's paternal half-sib family based on microsatellite markers. *Energy Procedia*, *47*, 196–203. https://doi.org/10.1016/j.egypro.2014.01.214

Souza, C. P. F., Ferreira, C. F., de Souza, E. H., Neto, A. R. S., Marconcini, J. M., da Silva Ledo, C. A., & Souza, F. V. D. (2017). Genetic diversity and ISSR marker association with the quality of pineapple fiber for use in industry. *Industrial Crops and Products*, *104*, 263–268. https://doi.org/10.1016/j.indcrop.2017.04.059

Statista. (2024). *Leading countries in pineapple production worldwide in 2022*. https://www.statista.com/statistics/298517/global-pineapple-production-by-leading-countries/

Terryana, R. T., Nugroho, K., & Lestari, P. (2020). Genetic diversity of sugar palm populations from Cianjur and Banten revealed by simple sequence repeat (SSR) markers. In *IOP Conference Series: Earth and Environmental Science* (Vol. 418, No. 1, p. 012038). IOP Publishing. https://doi.org/10.1088/1755-1315/418/1/012038

Valencia, L., & Alcasid, C. (2014). Morpho-genetic characterization of cultivated and wild relatives of pineapple in the Philippines. *Asian Academic Research Journal of Multidisciplinary*, *1*(27), 598–618.

Vanijajiva, O. (2012). Assessment of genetic diversity and relationships in pineapple cultivars from Thailand using ISSR marker. *Journal of Agricultural Technology*, *8*(5), 1829–1838.

Vijayan, K. (2005). Inter simple sequence repeat (ISSR) polymorphism and its application in mulberry genome analysis. *International Journal of Industrial Entomology*, *10*(2), 79–86.

Wang, J.-S., He, J.-H., Chen, H.-R., Chen, Y.-Y., & Qiao, F. (2017). Genetic diversity in various accessions of pineapple [*Ananas comosus* (L.) Merr.] using ISSR and SSR markers. *Biochemical Genetics*, *55*, 347–366. https://doi.org/10.1007/s10528-017-9803-z

Yeh, F. C., Boyle, T., Ye, Z., Xiyan, J. M., & Ye, Z. H. (1999). *POPGENE version 1.31: Microsoft Window-based freeware for population genetic analysis*. https://www.scienceopen.com/document?vid=02bb9771-6f17-47fa-8668-b71a53982e87

Zulfahmi. (2013). Penanda DNA untuk analisis genetik tanaman [DNA markers for plant genetic analysis]. *Jurnal Agroteknologi*, *3*(2), 41–52.

Pertanika J. Trop. Agric. Sci. 47 (4): 1101 - 1107 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

*Case Study*

# Feline Idiopathic Cystitis (FIC) in a Mixed Breed Cat: Case Study in Malaysia

**Syamira Syazuana Zaini*, Amir Shauqi Ahmad Sukri and Azalea Hani Othman**

*Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

## ABSTRACT

Feline idiopathic cystitis (FIC) is a common medical condition covering Feline Lower Urinary Tract Disease (FLUTD) in cats reported in many countries. However, there is a lack of prevalence studies reported in Malaysia. In this case report, a case of an FIC cat was presented at the University Veterinary Hospital, Universiti Putra Malaysia. The seven-month-old male mixed-breed cat had primary complaints of urine incontinence and haematuria. A thorough physical examination and diagnostic workup ruled out other causes, leading to the diagnosis of FIC. Based on the history provided by the owner, two weeks prior to the inappropriate urination, the cat moved into a new home, and the owner noticed that the cat appeared stressed and frequently urinated outside the litter box. In addition, the owner mentioned that dog bark noises were heard in the room where the cat was housed. Therefore, behavioural therapy (e.g., reconstitute the cat's home environment and increase the interaction between the owner and the cat) was also conducted as part of the medical intervention in this case. The owner reported no further recurrence of clinical signs. Thus, a complete recovery was achieved with a good prognosis.

*Keywords*: Behaviour therapy, cat, feline idiopathic cystitis, Malaysia, stress

ARTICLE INFO

*Article history:*
Received: 09 January 2024
Accepted: 04 March 2024
Published: 27 September 2024

DOI: https://doi.org/10.47836/pjtas.47.4.03

*E-mail addresses*:
syamirasz@upm.edu.my (Syamira Syazuana Zaini)
shauqi097@gmail.com (Amir Shauqi Ahmad Sukri)
azalea@upm.edu.my (Azalea Hani Othman)
* Corresponding author

## INTRODUCTION

Feline idiopathic cystitis (FIC) is a bladder inflammation caused by unknown causes (Jones, 2009). The clinical signs of FIC are mostly similar to those of Feline Lower Urinary Tract Disease (FLUTD), such as haematuria, dysuria, pollakiuria, stranguria, periuria, and vocalisation when

ISSN: 1511-3701
e-ISSN: 2231-8542

urinating (Defauw et al., 2011). There are several risk factors relating to FIC: male, overweight, young to middle-aged cat and stress-causing events (He et al., 2022; Jones, 2009).

According to Hostutler et al. (2005), FIC is the most common covering about 55-69% of FLUTD cases, as reported in the United States of America (USA) (Lekcharoensuk et al., 2001), Germany (Dorsch et al., 2014), South Korea (Kim et al., 2018), Thailand (Piyarungsri et al., 2020), and Indonesia (Nururrozi et al., 2020). However, there is still a lack of FIC prevalence studies in Malaysia, which could be assumed to be as high as reported in other countries.

## CASE PRESENTATION

A seven-month-old male mixed-breed cat was presented to the University Veterinary Hospital (UVH), Universiti Putra Malaysia (UPM), with primary complaints of urinary incontinence and blood-streaked urine. According to the owner, they had moved to a new home two weeks prior to the medical issue, where the cat seemed stressed and frequently showed inappropriate urination (e.g., urinating outside of the litter box). Besides, the owner also mentioned frequent dog barking near the cat's room.

### Physical and Laboratory Evaluation

Upon physical examination, the cat's heart rate was within normal range (160 bpm), mild hypothermia (T: 37.2°C) and tachypnoea (respiratory rate: 96 bpm), which could be due to pain, anxiety and/or stress. Abdominal palpation revealed a small urinary bladder; the physical examination was unremarkable.

Routine haematology and serum biochemistry were largely unremarkable except for mild neutropenia ($2.03 \times 10^9$/L, normal range: 2.5–12.5). In serum biochemistry results, there was a high level of alkaline phosphatase (178 U/L, normal range: <80), mild hyperalbuminaemia (42.5 g/L, normal range: 25–40) and hyperproteinaemia (76.8 g/L, normal range: 55–75).

Cystocentesis was performed, and the collected urine was sent for urinalysis, urine smear and urine culture. The urine appeared pale reddish with a turbidity of 3+. The chemical properties of the urine showed proteinuria at 3+. Microscopically, there were numerous erythrocytes and about 10–15 leukocytes. Bacterial culture yielded no growth.

An abdominal radiograph and ultrasound revealed a small urinary bladder with no urethral abnormalities and urolith (Figure 1). There was mild thickening of the bladder wall, an irregular pattern at the distal part of the bladder wall, and snowflake echogenicity in the urinary bladder (Figure 2).

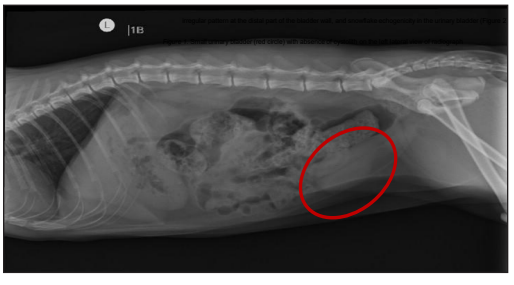


*Figure 1*. Small urinary bladder (red circle) with absence of cystolith on the left lateral view of radiograph

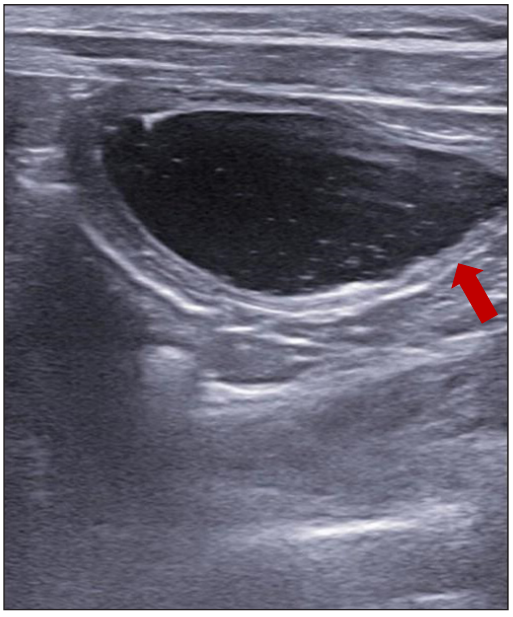


*Figure 2*. Irregular pattern at the distal part of the bladder wall (red arrow) and snowflake echogenicity in the bladder

## Behavioural History

According to the owner, they had moved to a new house two weeks prior to the inappropriate urination incident. At the previous house, the cat stayed with another senior cat, but the owner noticed no conflict. In the present house, there was only one cat (the patient), and the cat was placed in a room where all the basic resources (e.g., litter box, resting place, water and feeding bowl) were provided. The owner also reported that a dog lived in a nearby apartment, exactly next to the cat's room. The dog barked frequently, and the noise was significant. Therefore, a number of environmental stressors could be described for this cat, including moving to a novel environment, noise from dog barking, and restricted movement.

## Diagnosis

Based on the medical investigation, no other potential causes of FLUTD (e.g., urolithiasis, urethral plug, and bacterial cystitis) led to the diagnosis of FIC.

The behavioural history suggested that the cat had likely experienced stress due to a few environmental factors. Plus, moving to a novel environment, which in this case moved to a new house, could have restricted the cat from expressing its normal behaviours or 'coping' with potential stressors that might arise. In addition, a new environment, where the dog was barking consistently next to the cat's room could have made the cat feel threatened, and the stressor was unavoidable.

## Treatment

### *Medical Management*

On the first visit, the cat was treated with N-acetyl D-glucosamine (Cystaid®, VetPlus Ltd., United Kingdom) as a glycosaminoglycans (GAG) supplement, serratiopeptidase (Danzen®, Dynapharm Sdn. Bhd., Malaysia) as an anti-inflammatory, and amoxicillin + clavulanic acid (Augmentin™, GlaxoSmithKline, France) as a broad-spectrum antibiotic for two weeks.

A follow-up visit was scheduled for the cat, and according to the owner, the cat's stranguria persisted. Besides Cystaid® and Danzen®, the veterinarian in charge added diazepam (Duopharma Sdn. Bhd., Malaysia) and meloxicam (Pharmaniaga Manufacturing Berhad,

Malaysia) for urethral muscle relaxant and pain management.

### *Behaviour Therapy*

Since a few potential environmental stressors that led to this cat's medical issue were identified, so a few alternatives were discussed. The owner was instructed to move the cat to a room or area where the noise from dog barking could be minimised to reduce the stress caused by dog barking. In the potential new area, the basic environmental resources should be instituted, such as comfortable bedding for rest and sleep, a clean litter tray, a feed and water bowl and a hiding place. The owner was also advised to increase interaction with the cat, such as play, as this could encourage a positive human-cat relationship and reduce anxiety.

### *Progress and Prognosis*

The owner was very compliant in following management and behavioural advice. There was no further complaint about the cat's inappropriate urination, and this case was considered to have a good prognosis.

## DISCUSSION

FIC is an inflammation of the bladder due to unknown causes (Jones, 2009), which is only diagnosed when no agents or causes are identified after several diagnostic workups (Hostutler et al., 2005). FIC can be both obstructive and non-obstructive. The clinical signs of FIC are similar to FLUTD, such as haematuria, dysuria, pollakiuria, stanguria, periuria, vocalisation when urinating, and over-grooming of the genital area (Defauw et al., 2011). A study indicates several risk factors relating to FIC, such as male, overweight, fed with a dry diet only, young to middle-aged cats and stress-causing events (He et al., 2022; Jones, 2009).

Hostutler et al. (2005) stated that FIC is the most common, covering about 55-69% of FLUTD cases, such as studies on the prevalence of FLUTD caused by FIC ranging from 55-66% reported in the USA (Lekcharoensuk et al., 2001), Germany (Dorsch et al., 2014), South Korea (Kim et al., 2018), Thailand (Piyarungsri et al., 2020), and Indonesia (Nururrozi et al., 2020). However, there is a lack of study or data on the prevalence of FIC in Malaysia, which could be assumed as high as reported in other countries. Therefore, further study in this field is needed as this could give an idea of how we can manage it well, especially from both human and cat perspectives.

Currently, the pathophysiology of FIC is still unclear. There are a few hypotheses on the development of FIC. Comprehensive theories suggest that FIC is caused by changes in the nervous system of cats and their inability to cope with environmental stress (He et al., 2022) and that cats with FIC have smaller adrenal glands and a blunted stress response and produce higher levels of catecholamines than others without FIC (He et al., 2022). It causes changes in the interaction between the neuron and the bladder, aggravated by noxious substances in the urine. It may be increased further by altered interactions with the bladder's

protective GAG layer (Forrester & Towell, 2015). This response is called neurogenic inflammation (Buffington et al., 1996). The neurogenic inflammation begins with the stimulation of the C-fibres via central or local triggers. It can result in the release of neuropeptides such as substance P, which can cause pain, vasodilation of intramural blood vessels, increased vascular and bladder-wall permeability, submucosal oedema, smooth muscle contraction, and mast cell degranulation (Buffington et al., 1996; Chew & Buffington, 2013). Mast cell degranulation produces several inflammatory mediators, such as histamine, heparin, serotonin, cytokines, and prostaglandins, which can exacerbate the effects of the C-fibres. Many of the alterations observed in FIC can thus be explained by C-fibre stimulation and the ongoing neurogenic inflammation.

FIC is often difficult for clients to comprehend because, in many cases, the underlying cause may not be identified. It led to the difficulty of diagnosing FIC as the diagnosis will be announced once other potential differential diagnoses (e.g., urolithiasis, urinary tract infection, and urethral stricture) are ruled out (Hostutler et al., 2005). It is thought to be significantly influenced by environmental stress, for example, conflict in multi-cat households, moving to a novel environment or environmental changes such as construction work, bad weather, the arrival of new family members or pets, improper placement and cleanliness of the litter box, owner stress, a sudden change in diet, or a change in the cat's access to the outside (Cameron et al., 2004; Jones, 2009).

Since stress factors are crucial in the development of FIC, prevention by management is the best method to reduce the recurrence (Westropp & Buffington, 2004). It can be achieved through multimodal environmental modification (MEMO) (He et al., 2022; Lund & Eggertsdóttir, 2019). MEMO can substantially reduce lower urinary tract symptoms, fearfulness, and nervousness in cats with idiopathic cystitis. According to Buffington et al. (2006), there are five essential elements for a healthy feline environment in MEMO. The first pillar is to offer a safe environment for cats by providing specific hiding spots such as cat trees and cat tunnels. The second pillar entails providing multiple and distinct supplies for the food and drink bowl, litter box, and scratching area. It may help to mediate inter-cat conflicts by reducing competition for resources. The third pillar encourages play and predatory behaviour in cats by utilising interactive cat toys that imitate the cat's natural hunting impulse. The fourth pillar is owner must provide a good, consistent human-cat social connection: each cat in a multi-cat household should receive personalised attention without interference from other cats. Finally, the fifth pillar is to develop an environment that values the cat's sense of smell. It can be accomplished by minimising strong odours, detergents, and scented litter, which interfere with the cat's sensory awareness and odours associated with its regular environment (Ellis et al., 2013).

In the current case, the cat was successfully managed medically, plus

behavioural therapy. As described, a few potential environmental stressors caused FIC in the cat, such as moving to a new house, encountering an unfamiliar place, and experiencing loud noise from the dog's barking. The MEMO five elements were applied accordingly based on the issues identified. For example, in the current case, the owner was advised to move the cat to a room or area where the noise from the dog barking could be minimised. In the potential new area, the basic environmental resources were instituted, such as comfortable bedding for rest and sleep, a clean litter tray, a feed and water bowl, and a hiding place. The owner was also advised to increase interaction with the cat, such as play, as this could encourage a positive human-cat relationship and reduce anxiety. As the owner was very compliant in following management and behavioural advice, there is no further complaint about the cat's inappropriate urination. Therefore, in future FLUTD, due to high suspicion of FIC, the case is encouraged to be managed in medical and behavioural therapy.

## CONCLUSION

In the current case, inappropriate urination in a cat was highly suspected due to FIC, and it was treated using both medical and behavioural therapy. Inappropriate urination is required when investigating the potential cause of the issue as it helps the institution develop a proper plan for managing the issue successfully. Plus, it could improve the cat's well-being and reduce the chance of recurrence.

## ACKNOWLEDGEMENTS

The authors thank the University Veterinary Hospital, Universiti Putra Malaysia (UPM), and the laboratory staff of the Veterinary Laboratory Service Unit, UPM, for their tremendous work, quality testing, and services in managing this case. Thanks to the Research Management Centre (RMC), UPM, for supporting the journal publication fund.

## REFERENCES

Buffington, C. A. T., Chew, D. J., & DiBartola, S. P. (1996). Interstitial cystitis in cats. *Veterinary Clinics of North America: Small Animal Practice*, *26*(2), 317–326. https://doi.org/10.1016/S0195-5616(96)50212-3

Buffington, C. A. T., Westropp, J. L., Chew, D. J., & Bolus, R. R. (2006). Clinical evaluation of multimodal environmental modification (MEMO) in the management of cats with idiopathic cystitis. *Journal of Feline Medicine and Surgery*, *8*(4), 261–268. https://doi.org/10.1016/j.jfms.2006.02.002

Cameron, M. E., Casey, R. A., Bradshaw, J. W. S., Waran, N. K., & Gunn-Moore, D. A. (2004). A study of environmental and behavioural factors that may be associated with feline idiopathic cystitis. *Journal of Small Animal Practice*, *45*(3), 144-147. https://doi.org/10.1111/j.1748-5827.2004.tb00216.x

Chew, D., & Buffington, C. A. T. (2013). *Pandora syndrome: It's more than just the bladder*. https://justcatsonline.com/wp-content/uploads/2023/05/Pandora_Syndrome.pdf?ver

Defauw, P. A., Van de Maele, I., Duchateau, L., Polis, I. E., Saunders, J. H., & Daminet, S. (2011). Risk factors and clinical presentation of cats with feline idiopathic cystitis. *Journal of Feline Medicine and Surgery*, *13*(12), 967-975. https://doi.org/10.1016/j.jfms.2011.08.001

Dorsch, R., Remer, C., Sauter-Louis, C., & Hartmann, K. (2014). Feline lower urinary tract disease in a German cat population: A retrospective analysis of demographic data causes and clinical signs. *Tierärztliche Praxis Ausgabe K: Kleintiere / Heimtiere*, *42*(4), 231–239. https://doi.org/10.1055/s-0038-1623769

Ellis, S. L. H., Rodan, I., Carney, H. C., Heath, S., Rochlitz, I., Shearburn, L. D., Sundahl, E., & Westropp, J. L. (2013). AAFP and ISFM feline environmental needs guidelines. *Journal of Feline Medicine and Surgery*, *15*(3), 219-230. https://doi.org/10.1177/1098612X13477537

Forrester, S. D., & Towell, T. L. (2015). Feline idiopathic cystitis. *Veterinary Clinics of North America: Small Animal Practice*, *45*(4), 783-806. https://doi.org/10.1016/j.cvsm.2015.02.007

He, C., Fan, K., Hao, Z., Tang, N., Li, G., & Wang, S. (2022). Prevalence, risk factors, pathophysiology, potential biomarkers and management of feline idiopathic cystitis: An update review. *Frontiers in Veterinary Science*, *9*, 900847. https://doi.org/10.3389/fvets.2022.900847

Hostutler, R. A., Chew, D. J., & DiBartola, S. P. (2005). Recent concepts in feline lower urinary tract disease. *Veterinary Clinics of North America: Small Animal Practice*, *35*(1), 147-170. https://doi.org/10.1016/j.cvsm.2004.08.006

Jones, A. (2009). Feline Lower Urinary Tract Disease (FLUTD): An overview. *Veterinary Nursing Journal*, *24*(3), 21-24. https://doi.org/10.1080/17415349.2009.11013073

Kim, Y., Kim, H., Pfeiffer, D., & Brodbelt, D. (2018). Epidemiological study of feline idiopathic cystitis in Seoul, South Korea. *Journal of Feline Medicine and Surgery*, *20*(10), 913–921. https://doi.org/10.1177/1098612X17734067

Lekcharoensuk, C., Osborne, C. A., & Lulich, J. P. (2001). Epidemiologic study of risk factors for lower urinary tract diseases in cats. *Journal of the American Veterinary Medical Association*, *218*(9), 1429–1435. https://doi.org/10.2460/javma.2001.218.1429

Lund, H. S., & Eggertsdóttir, A. V. (2019). Recurrent episodes of feline lower urinary tract disease with different causes: possible clinical implications. *Journal of Feline Medicine and Surgery*, *21*(6), 590-594. https://doi.org/10.1177/1098612X18783839

Nururrozi, A., Yanuartono, Y., Sivananthan, P., & Indarjulianto, S. (2020). Evaluation of lower urinary tract disease in the Yogyakarta cat population, Indonesia. *Veterinary World*, *13*(6), 1182–1186. https://doi.org/10.14202/vetworld.2020.1182-1186

Piyarungsri, K., Tangtrongsup, S., Thitaram, N., Lekklar, P., & Kittinuntasilp, A. (2020). Prevalence and risk factors of feline lower urinary tract disease in Chiang Mai, Thailand. *Scientific Reports*, *10*, 196. https://doi.org/10.1038/s41598-019-56968-w

Westropp, J. L., & Buffington, C. A. T. (2004). Feline idiopathic cystitis: Current understanding of pathophysiology and management. *Veterinary Clinics of North America: Small Animal Practice*, *34*(4), 1043-1055. https://doi.org/10.1016/j.cvsm.2004.03.002

Pertanika J. Trop. Agric. Sci. 47 (4): 1109 - 1120 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Serological Investigation of Aujeszky's Disease Between 2019 and 2021 in Peninsular Malaysia

**Hong Xia Li[1], Michelle Wai Cheng Fong[1], Nor Yasmin Abdul Rahaman[2,3], Suet Ee Low[1], Jia Xin Lee[4], Eric Heng Chow Cheah[4], Kok Yen Kam[4], Raquel Li Hui Yong[4] and Peck Toung Ooi[1]***

*[1]Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
*[2]Department of Veterinary Laboratory Diagnostics, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
*[3]Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
*[4]Animal Health Division, Boehringer Ingelheim Malaysia Sdn. Bhd., 50490, Kuala Lumpur, Malaysia*

## ABSTRACT

Aujeszky's disease (AD) is a common disease that has spread worldwide. Various porcine viral diseases exist in Malaysia, where AD is the most common viral endemic disease in the country. The serological status of AD in Peninsular Malaysia was reported prior to 2018, but information after that date is very limited. Hence, our study investigated AD's serological status in Peninsular Malaysia pig farms based on commercial samples submitted to the Faculty of Veterinary Medicine, Universiti Putra Malaysia, between 2019 and 2021. In this study, 2,780 serum samples were taken from 61 farms, and an enzyme-linked immunosorbent assay (ELISA) test was performed using the IDEXX Pseudorabies Virus gpl Antibody Test Kit for AD serology diagnosis. The results showed that the overall seropositive rate of Aujeszky disease virus (ADV) was 1.51% (42/2,780), which dropped from 2.62% (23/879) in 2019 to 0.53% (5/937) in 2020 and 1.45% (14/964) in 2021. In addition, 18.03% (11/61) of the 61 farms that submitted samples were infected with AD. The results indicate that AD still exists in Peninsular Malaysia, and some farms are at risk from the disease. Further analysis suggested that the quarterly seroprevalence of ADV may also be related to region. This study provides serological

ARTICLE INFO

*Article history:*
Received: 01 February 2024
Accepted: 18 March 2024
Published: 27 September 2024

DOI: https://doi.org/10.47836/pjtas.47.4.04

*E-mail addresses*:
Lihongxia12321@gmail.com (Hong Xia Li)
michelle@upm.edu.my (Michelle Wai Cheng Fong)
noryasmin@upm.edu.my (Nor Yasmin Abdul Rahaman)
vynter_ee0914@hotmail.com (Suet Ee Low)
jia-xin.lee@boehringer-ingelheim.com (Jia Xin Lee)
eric.cheah@boehringer-ingelheim.com (Eric Heng Chow Cheah)
kok-yen.kam@boehringer-ingelheim.com (Kok Yen Kam)
raquel.yong@boehringer-ingelheim.com (Raquel Li Hui Yong)
ooi@upm.edu.my (Peck Toung Ooi)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

data on AD in Peninsular Malaysia, which helps to understand the prevalence and serological status of the disease.

*Keywords*: Aujeszky's disease, ELISA, Peninsular Malaysia, pig farm, serological status

## INTRODUCTION

Aujeszky's disease (AD) is one of the most common pig viral diseases, which causes significant economic losses to the pig industry. AD first appeared in the United States in 1813, and it was first characterized in several animals by scientist Aladar Aujeszky in 1902, so it was named Aujeszky's disease (Aujeszky, 1902). AD, called pseudorabies (PR), is highly infectious. The causative agent of AD is the Aujeszky disease virus (ADV), which is also known as pseudorabies virus (PRV) or Suid herpesvirus 1 (SuHV1) (Freuling et al., 2017). ADV belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and genus *Varicellovirus* (Davison, 2010). ADV is a double-stranded, enveloped DNA virus, and the total length of the ADV genome is approximately 150 kb. The virus has 11 glycoproteins, namely glycoprotein B (gB), glycoprotein C (gC), glycoprotein D (gD), glycoprotein E (gE), glycoprotein G (gG), glycoprotein H (gH), glycoprotein I (gI), glycoprotein K (gK), glycoprotein L (gL), glycoprotein M (gM), and glycoprotein N (gN) (Mettenleiter, 2000). The gE is also called glycoprotein I (gpI), the latter being very old terminology (Low et al., 2018).

The only reservoirs and natural hosts of ADV are members of the family Suidae (domestic and wild pig), and pigs at every age are susceptible to ADV (Müller et al., 2011). Besides pigs, ADV can also infect many animals, including carnivores, ruminants, and rodents (Konjević et al., 2023). However, only pigs can survive among all animals susceptible to AD (Delva et al., 2020).

AD is mainly transmitted through direct nose-to-nose contact between pigs. In addition, ADV can be transmitted indirectly through aerosols, semen, and fomites (Aznar et al., 2022). The virus can also be transmitted vertically across the placenta (Ukhovskyi et al., 2022). Clinical signs of disease depend on the age of the pig, infection dose, virus strain, and health status (Pomeranz et al., 2005; Szczotka-Bochniarz et al., 2016). Piglets mainly exhibit severe neurological symptoms and fatal encephalitis, with mortality approaching 100%. Fattening pigs' most prominent clinical symptoms are respiratory symptoms, slow growth, and mortality, usually less than 5% (Chen et al., 2022; Nauwynck, 1997). Sows infected with ADV usually have abortions, give birth to mummified fetuses or dead piglets, and are infertile (Baskerville, 1981). Usually, the disease is not fatal in older pigs (Zuckermann, 2000).

In the 1970–1980s, the first widespread outbreak of ADV occurred in swine herds worldwide (Liu et al., 2022). Although AD has been eradicated in domestic pigs in many countries such as Germany, the United States, New Zealand, Canada, the United Kingdom, and Sweden in the late 1990s, it is still prevalent in Europe, South America,

and Asia (Aznar et al., 2022; Sun et al., 2016). In Malaysia, AD was first reported in 1976 (Lee et al., 1979), and AD was declared endemic in 1984 (Too, 1997). A study in 2016 found that AD field strains still exist in Malaysia (Low et al., 2018). Although AD vaccination is not mandatory in Malaysia's Department of Veterinary Services, it is currently commonly performed in pig farms.

Several modified live vaccines and inactivated vaccines have been approved in Malaysia to prevent and control AD. Most AD vaccines used on farms in the country are gE-deleted vaccines. Such marker vaccines can differentiate between infected and vaccinated animals (DIVA) (Delva et al., 2020; Freuling et al., 2017; Ukhovskyi et al., 2022). Therefore, gE antibodies produced by the AD field virus can be detected in infected animals by the enzyme-linked immunosorbent assay (ELISA) test but not in vaccinated (uninfected) animals (Mettenleiter, 2020; Wang et al., 2019).

Serological surveys were conducted in Peninsular Malaysia in 2016 to better control and prevent AD, but the disease's serological prevalence in recent years remains unknown. Therefore, this study aims to investigate the serological status of AD between 2019 and 2021 in Peninsular Malaysia and detect gE antibodies by ELISA test.

## MATERIALS AND METHODS

### Sample Collection

Farmers provided verbal informed consent for the collection of serum samples and AD serological diagnosis. Farmers submitted pig serum samples to the Faculty of Veterinary Medicine, Universiti Putra Malaysia. A total of 2,780 serum samples from 61 farms met the criteria of this study from January 2019 to December 2021. In general, at least 20 serum samples were submitted from each farm, comprising 1–6 weeks, 7–12 weeks, 12–20 weeks, gilts, and sows. At least 4 samples were submitted for each listed age group. Each serum sample was collected the day after blood collection, then stored and transported to the laboratory at -80°C. The monthly test results are summarized and sorted by region.

### Region Categorization

All farms were categorized according to location into four categories (Perak, Johor, Penang, Selangor, and Malacca regions). Due to the small number of farms and samples in the Selangor and Malacca regions, they are classified as one region.

### Serological Detection

Detection of antibodies towards the gE/gpI antigen of ADV in serum samples by ELISA test using the Pseudorabies Virus gpI Antibody Test Kit (IDEXX Laboratories, Inc., USA) according to the manufacturer's instructions. The kit can distinguish infected pigs from vaccinated pigs. The immune response produced by pigs vaccinated with the gE/gpI deletion vaccine does not contain gE/gpI. At the same time, gE/gpI antibodies will be detected in pigs infected with AD field viruses (Low et al., 2018). The gE/gpI deleted vaccine was used to immunize pigs against AD pig farms in Malaysia.

Therefore, when antibodies against gE are detected in the serum of pigs vaccinated with the gE/gpI deleted vaccine, it indicates that the pigs are infected with AD field virus (van Oirschot et al., 1990). The ELISA test is the most widely used diagnostic method to detect the presence of ADV antibodies. The assay can also screen many serum samples in a short time, and it has higher sensitivity and specificity.

The serological status of the sample was determined by the sample-to-negative ratio (S/N) value. S/N ≤ 0.6 is considered positive, which indicates that the sample is infected with ADV field strains. 0.6 < S/N < 0.7 is considered suspect. S/N ≥ 0.7 is considered a negative result for the sample.

## RESULTS AND DISCUSSION

In this study, 2,780 serum samples were submitted from four regions in Peninsular Malaysia, including 879 samples in 2019, 937 samples in 2020, and 964 samples in 2021. The results of the ELISA test show that the overall positive seroprevalence of samples was 1.51% during 2019–2021. The seroprevalence of samples from 2019 to 2021 was 2.62% (23/879), 0.53% (5/937), and 1.45% (14/964), respectively. In addition, different regions have different seroprevalences of samples in 2019–2021. None of the samples tested positive in the Perak region. In contrast, the seroprevalence of samples was 5.92% in the Johor region, 0.27% in the Penang region, and 3.77% in the Selangor and Malacca regions (Table 1).

These serum samples were collected from 61 farms, including 20 in 2019, 22 in 2020, and 19 in 2021. The percentage of infected farms is not the same from 2019 to 2021. Eleven (18.03%) farms had positive serum samples detected in these three years. Six (30%) farms were infected with AD in 2019, two (9.09%) farms in 2020, and three (15.79%) farms in 2021. In addition, the percentage of infected farms in different

Table 1
*Percentage of seropositive samples in different regions across years between 2019 and 2021 in Peninsular Malaysia*

| Region | Percentage of seropositive samples (%) | | | |
|---|---|---|---|---|
| | 2019 | 2020 | 2021 | Overall |
| Perak | 0 | 0 | 0 | 0 |
| | 0/527 | 0/568 | 0/466 | 0/1,561 |
| Johor | 16.90 | 10 | 2.89 | 5.92 |
| | 12/71 | 4/40 | 9/311 | 25/422 |
| Penang | 0 | 0 | 1.28 | 0.27 |
| | 0/107 | 0/188 | 1/78 | 1/373 |
| Selangor and Malacca | 6.32 | 0.71 | 3.67 | 3.77 |
| | 11/174 | 1/141 | 4/109 | 16/424 |
| Total | 2.62 | 0.53 | 1.45 | 1.51 |
| | 23/879 | 5/937 | 14/964 | 42/2,780 |

*Note*. The number of seropositive samples/The number of total samples

regions also varied in 2019–2021. No farms were infected with AD in the Perak region, while pig farms are highly affected by the disease in the Selangor and Malacca regions, pig farms are moderately affected in the Johor region, and pig farms are lowly affected in the Penang region (Table 2).

The seroprevalence also varies among different age groups. The seroprevalence of samples was 2.37% in piglets aged 1–6 weeks, 0.47% in weaned piglets aged 7–12 weeks, 0.34% in fattening pigs aged 13–20 weeks, 0.88% in gilts, and 3.16% in sows (Table 3). Based on this study, it was observed that at 1–6 weeks of age, gilts, and sow herds were most likely to be seropositive. For breeding herds (gilts and sows), high seroprevalence may be

Table 2
*Percentage of infected farms in different regions across years between 2019 and 2021 in Peninsular Malaysia*

| Region | Percentage of infected farms (%) | | | |
|---|---|---|---|---|
| | 2019 | 2020 | 2021 | Overall |
| Perak | 0 | 0 | 0 | 0 |
| | 0/10 | 0/13 | 0/10 | 0/33 |
| Johor | 100 | 100 | 20 | 50 |
| | 2/2 | 1/1 | 1/5 | 4/8 |
| Penang | 0 | 0 | 50 | 10 |
| | 0/3 | 0/5 | 1/2 | 1/10 |
| Selangor and Malacca | 80 | 33.33 | 50 | 60 |
| | 4/5 | 1/3 | 1/2 | 6/10 |
| Total | 30 | 9.09 | 15.79 | 18.03 |
| | 6/20 | 2/22 | 3/19 | 11/61 |

*Note*. The number of infected farms/The number of total farms

Table 3
*Percentage of seropositive samples in different regions across age groups between 2019 and 2021 in Peninsular Malaysia*

| Region | Percentage of seropositive samples (%) | | | | |
|---|---|---|---|---|---|
| | 1–6 weeks | 7–12 weeks | 13–20 weeks | Gilts | Sows |
| Perak | 0 | 0 | 0 | 0 | 0 |
| | 0/289 | 0/63 | 0/339 | 0/187 | 0/383 |
| Johor | 7.23 | 3.23 | 1.15 | 0 | 14.56 |
| | 6/83 | 3/93 | 1/87 | 0/56 | 15/103 |
| Penang | 0 | 0 | 1.20 | 0 | 0 |
| | 0/82 | 0/82 | 1/83 | 0/44 | 0/82 |
| Selangor and Malacca | 7.45 | 0 | 0 | 5.77 | 6.25 |
| | 7/94 | 0/97 | 0/85 | 3/52 | 6/96 |
| Total | 2.37 | 0.47 | 0.34 | 0.88 | 3.16 |
| | 13/548 | 3/635 | 2/594 | 3/339 | 21/664 |

*Note*. The number of seropositive samples/The number of total samples

caused by the introduction of pigs from AD-infected herds or the failure of vaccine immunization (Siegel & Weigel, 1999). The higher seroprevalence in piglets aged 1–6 weeks is most likely caused by the influence of maternally derived antibodies (MDA). When MDA levels rise during vaccination, immunization effectiveness declines, and the development of active immunity is also interfered with by MDA (Stegeman, 1995). Another reason might be that sows are infected with ADV during pregnancy, causing newborn piglets to be infected with ADV from the placenta (Laval & Enquist, 2020).

This study's overall positive rate of ADV gE antibodies dropped from 2.62% in 2019 to 0.53% in 2020 and 1.45% in 2021. This result was lower than 4.25% (49/1154) in 2016 (Low et al., 2018). Moreover, the percentage of AD-infected farms dropped from 30% in 2019 to 9.09% in 2020 and 15.79% in 2021. Although the seroprevalence rates in different years are irregular, the overall data shows that the field infections of AD are on a downward trend in Peninsular Malaysia. It may be related to the coronavirus disease (COVID-19) outbreaks in 2020 and African swine fever (ASF) in 2021 in Malaysia. Farmers have started to practice restricted movement by strengthening biosecurity measures and feeding management to prevent COVID-19 and ASF from invading the farm (Khoo et al., 2021).

The obtained data indicated that the seroprevalence rate of ADV infection in pigs in different regions is different in Peninsular Malaysia, which may be due to different factors such as farm setup, husbandry practice, vaccination and concurrent infections in this serological survey: Perak region had the largest number of samples and farms, no seropositive samples were detected, and no farms were infected with the AD field virus during these three years. It may be attributed to good husbandry and biosecurity practices on farms, as well as regular vaccination of pig herds with highly effective AD vaccines. Although no samples were found to be seropositive for field-type AD infection in this investigation, it does not mean that the region is completely free of the threat of AD. Therefore, farmers should also remain concerned about this disease.

Compared to other regions, the Johor region has the highest overall seropositivity rate, and half of farms are infected with the AD field virus. However, with only a limited number of farms and samples collected from this region, it was not able to represent the whole situation in the region. This region had high levels of seroprevalence in 2019, which may be largely due to internal factors on farms causing AD infections. Different factors, such as farm husbandry, vaccinations, and biosecurity measures, all contributed to farm disease conditions (Ukhovskyi et al., 2022). Although the seroprevalence of AD was very high in 2019, the situation has improved greatly in 2020 and 2021.

The AD field infection challenge for the Penang region is very low, and only one farm was affected. It indicates that AD was stable and did not challenge the farms. According to the results, there was no AD field virus

infection in the Penang region in 2019 and 2020, while only one farm was infected by ADV in 2021. Overall, the spread of AD field viruses in the Penang region was relatively low, consistent with the results of the serological survey on AD in the Penang region in 2016 (Low et al., 2018).

The number of ADV-infected farms is the highest in the Selangor and Malacca regions. The AD field virus is regional and common in this region, most likely due to the high pig farm density in these two regions. ADV can also spread through the air over long distances between farms, more than 10 km (Casal et al., 1997). Moreover, according to serology results in the Selangor and Malacca regions, antibodies against the AD field virus were detected in piglets, gilts, and sows. The external introduction of ADV, which carries breeding pigs (sows and gilts) and semen, is the main source of farm infection with AD (Song et al., 2017). Infected sows may continue to spread and excrete ADV on the farm, which may expose naive porkers or sows to ADV infection. Therefore, to reduce the high seroprevalence of AD and prevent farms from being affected by the disease, farmers must strictly implement biosecurity measures and strengthen the management of farm husbandry. Further investigation found that farms with biosecurity measures such as vehicle dip, isolation and holding room, and foot dip have a reduced risk of ADV infection. It was also observed that open-type farms and farms surrounded by neighboring farms are more susceptible to ADV challenges.

The data show that the seroprevalence of ADV may be correlated with quarter and region. Due to the impact of COVID-19 and ASF, staff access to farms has been restricted, and many farms have closed down, making it difficult to collect samples for this study. Therefore, in some quarters, we did not collect samples. However, it can be seen from the limited data that the seroprevalence rate was 5.11% in the third quarter (Q3) and 2.01% in the second quarter (Q2) of 2019. In 2020, the seroprevalence rate in the third quarter (Q3) was 0.95%. The seroprevalence rate in the fourth quarter (Q4) was 6.78%, and it was the highest in 2021, followed by 2.87% and 0.58% in the first (Q1) and third quarters (Q3), respectively (Table 4). The overall data results indicate that all cases will increase in the fourth quarter (Q4) of 2021. From 2019 to 2021, no samples were detected positive for seropositivity in the Perak region. In the Johor region, the seroprevalence rate was the highest in the third quarter (Q3), especially in 2019, with a seroprevalence rate of 16.90%. No serum samples were detected positive in the Penang region from the first quarter (Q1) of 2019 to the third quarter (Q3) of 2021, but 2.50% of the serum samples were positive until the fourth quarter (Q4) of 2021. In the Selangor and Malacca regions, the seroprevalence of ADV is irregular. There was a clear upward trend in the fourth quarter (Q4) of 2021, but it is worth noting that the serum samples in the first quarter (Q1) from 2019 to 2021 were seronegative (Figure 1). It can be found that the Perak region has not been affected by

ADV during the three-year period, while the seroprevalence in the Johor, Selangor, and Malacca regions is higher. There are some differences in seroprevalence rates among regions in different quarters. ADV infection rates vary by quarter and region, consistent with previous studies (Zheng et al., 2021). It shows that although the seroprevalence of ADV is very low, it is still difficult to eradicate the disease in Peninsular Malaysia. In addition, the seroprevalence rate of pigs infected with ADV is on the rise in the fourth quarter (Q4) of 2021. Therefore, pig farms also need to take preventive measures against the disease.

Many cases of human infection with ADV have been reported in recent years, especially in China. Furthermore, it has been demonstrated that ADV may invade the human central nervous system (CNS) and cause endophthalmitis and encephalitis in humans when infecting them through the eyes (Ai et al., 2018) and wounds (Yang et al., 2019). Although human cases of ADV infection occurred in China, its potential harm to human public health worldwide cannot be ignored. Through communication with farmers, it could be learned that in Malaysia, to prevent human infection with ADV, unauthorized people are required to stay away from the farm, and staff allowed to enter the farm are required to decontaminate and disinfect their hands, shoes, and clothing before leaving the farm. In addition, people who work with pigs (e.g., pig farmers, veterinarians, and slaughterers) are advised to protect themselves while working, avoid exposure to wounds, and avoid direct contact with infected pigs or their fluids.

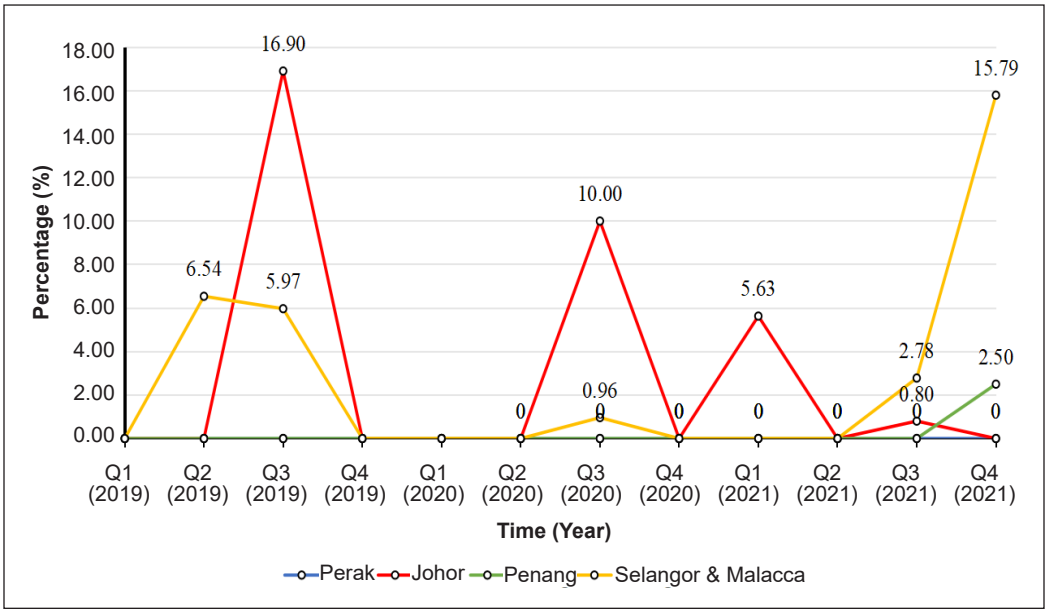



*Figure 1*. Percentage of seropositive samples in different quarters across regions
*Note*. Q1 = First quarter (from January to March); Q2 = Second quarter (from April to June); Q3 = Third quarter (from July to September); Q4 = Fourth quarter (from October month to December)

Table 4
*Percentage of seropositive samples in different quarters across regions between 2019 and 2021 in Peninsular Malaysia*

| Time | The regions of Peninsular Malaysia | | | | Total |
|---|---|---|---|---|---|
| | Perak | Johor | Penang | Selangor and Malacca | |
| January–March (Q1) 2019 | NA | NA | 0.00% 0/47 | NA | 0.00% 0/47 |
| April–June (Q2) 2019 | 0.00% 0/242 | NA | NA | 6.54% 7/107 | 2.01% 7/349 |
| July–September (Q3) 2019 | 0.00% 0/115 | 16.90% 12/71 | 0.00% 0/60 | 5.97% 4/67 | 5.11% 16/313 |
| October–December (Q4) 2019 | 0.00% 0/170 | NA | NA | NA | 0.00% 0/170 |
| Total 2019 | 0.00% 0/527 | 16.90% 12/71 | 0.00% 0/107 | 6.32% 11/174 | 13.22% 23/879 |
| January–March (Q1) 2020 | 0.00% 0/95 | NA | 0.00% 0/34 | 0.00% 0/37 | 0.00% 0/166 |
| April–June (Q2) 2020 | 0.00% 0/131 | NA | 0.00% 0/34 | NA | 0.00% 0/165 |
| July–September (Q3) 2020 | 0.00% 0/302 | 10.00% 4/40 | 0.00% 0/82 | 0.96% 1/104 | 0.95% 5/528 |
| October–December (Q4) 2020 | 0.00% 0/40 | NA | 0.00% 0/38 | NA | 0.00% 0/78 |
| Total 2020 | 0.00% 0/568 | 10.00% 4/40 | 0.00% 0/188 | 0.71% 1/141 | 0.53% 5/937 |
| January–March (Q1) 2021 | 0.00% 0/83 | 5.63% 8/142 | NA | 0.00% 0/54 | 2.87% 8/279 |
| April–June (Q2) 2021 | 0.00% 0/237 | 0.00% 0/44 | NA | NA | 0.00% 0/281 |
| July–September (Q3) 2021 | 0.00% 0/146 | 0.80% 1/125 | 0.00% 0/38 | 2.78% 1/36 | 0.58% 2/345 |
| October–December (Q4) 2021 | NA | NA | 2.50% 1/40 | 15.79% 3/19 | 6.78% 4/59 |
| Total 2021 | 0.00% 0/466 | 2.89% 9/311 | 1.28% 1/78 | 3.67% 4/109 | 1.45% 14/964 |
| Grand Total (2019–2021) | 0.00% 0/1561 | 5.92% 25/422 | 0.27% 1/373 | 3.77% 16/424 | 1.51% 42/2,780 |

*Note*. NA = Not applicable as no samples were submitted during that period; Q = Quarters

## CONCLUSION

This study investigated the serological prevalence of AD in Peninsular Malaysia during 2019–2021. The seroprevalence of AD varies between different regions. The Perak region is free of AD field virus infections. The seroprevalence of AD is the highest in the Johor region but might be biased due to sample size. The seroprevalence of AD in the Penang

region is relatively low, and the number of farms infected with AD is also small. The Selangor and Malacca regions have the highest number of AD-infected farms. In addition, the seroprevalence of ADV is also related to the quarter, with pigs being more susceptible to the disease in the third quarter (Q3). Overall, the exposure level to AD field viruses is low from 2019 to 2021. With the widespread use of highly effective AD vaccines, AD has been well controlled in pig farms in Peninsular Malaysia, so the next step should be to eradicate the disease in the country. This study contributes to a better understanding of the serological Investigation of AD in Peninsular Malaysia and provides basic information for the disease's prevention, control, and eradication.

## ACKNOWLEDGMENTS

The authors express gratitude to the Veterinary Clinical Laboratory and Virology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia, for providing help in this study.

## REFERENCES

Ai, J.-W., Weng, S.-S., Cheng, Q., Cui, P., Li, Y.-J., Wu, H.-L., Zhu, Y.-M., Xu, B., & Zhang, W.-H. (2018). Human endophthalmitis caused by pseudorabies virus infection, China, 2017. *Emerging Infectious Diseases*, *24*(6), 1087–1090. https://doi.org/10.3201/eid2406.171612

Aujeszky, A. (1902). *Über eine neue infektions krankheit bei haustieren* [About a new infectious disease in domestic animals]. Fischer.

Aznar, M. N., Bessone, F. A., Segurado, R., & Duffy, S. J. (2022). Assessment of an Aujeszky's disease control strategy in a highly prevalent pig farm based on systematic vaccination with an inactivated gE-negative marker vaccine. *Frontiers in Veterinary Science*, *9*, 852650. https://doi.org/10.3389/fvets.2022.852650

Baskerville, A. (1981). Aujeszky's disease: Recent advances and current problems. *New Zealand Veterinary Journal*, *29*(10), 183-185. https://doi.org/10.1080/00480169.1981.34837

Casal, J., Moreso, J. M., Planas-Cuchí, E., & Casal, J. (1997). Simulated airborne spread of Aujeszky's disease and foot-and-mouth disease. *Veterinary Record*, *140*(26), 672-676. https://doi.org/10.1136/vr.140.26.672

Chen, X., Li, H., Zhu, Q., Chen, H., Wang, Z., Zheng, L., Liu, F., & Wei, Z. (2022). Serological investigation and genetic characteristics of pseudorabies virus between 2019 and 2021 in Henan province of China. *Viruses*, *14*(8), 1685. https://doi.org/10.3390/v14081685

Davison, A. J. (2010). Herpesvirus systematics. *Veterinary Microbiology*, *143*(1), 52-69. https://doi.org/10.1016/j.vetmic.2010.02.014

Delva, J. L., Nauwynck, H. J., Mettenleiter, T. C., & Favoreel, H. W. (2020). The attenuated pseudorabies virus vaccine strain Bartha K61: A brief review on the knowledge gathered during 60 years of research. *Pathogens*, *9*(11), 897. https://doi.org/10.3390/pathogens9110897

Freuling, C. M., Müller, T. F., & Mettenleiter, T. C. (2017). Vaccines against pseudorabies virus (PrV). *Veterinary Microbiology*, *206*, 3-9. https://doi.org/10.1016/j.vetmic.2016.11.019

Khoo, C. K., Norlina, D., Roshaslinda, D., Siti Suraya Hani, M. S., Zunaida, B., Mohd Hasrul, A. H., Pauzi, N. A. S., Roslina, H., Faizah Hanim, M. S., & Leow, B. L. (2021). African swine fever in backyard pigs of Sabah state, East Malaysia,

2021. *Tropical Biomedicine*, *38*(4), 499-504. https://doi.org/10.47665/tb.38.4.095

Konjević, D., Sučec, I., Turk, N., Barbić, L., Prpić, J., Krapinec, K., Bujanić, M., Jemeršić, L., & Keros, T. (2023). Epidemiology of Aujeszky disease in wild boars (*Sus scrofa* L.) in Croatia. *Veterinary Research Communications*, *47*, 631-639. https://doi.org/10.1007/s11259-022-10017-6

Laval, K., & Enquist, L. W. (2020). The neuropathic itch caused by pseudorabies virus. *Pathogens*, *9*(4), 254. https://doi.org/10.3390/pathogens9040254

Lee, J. Y. S., Wilson, M. R., & Porey, R. H. C. (1979). The efficacy of an inactivated vaccine against pseudorabies in pigs and sheep. *Kajian Veterinar*, *11*, 58-64.

Liu, Q., Kuang, Y., Li, Y., Guo, H., Zhou, C., Guo, S., Tan, C., Wu, B., Chen, H., & Wang, X. (2022). The epidemiology and variation in pseudorabies virus: A continuing challenge to pigs and humans. *Viruses*, *14*(7), 1463. https://doi.org/10.3390/v14071463

Low, S. E., Ooi, P. T., Rahaman, N. Y. A., Tan, S. W., Cheah, Z. H., Cheah, E. H. C., Lim, E. P. Q., Yong, C. K. & Kam, K.Y. (2018). Serological survey of aujeszky's disease in Peninsular Malaysia in 2016. *Advances in Animal and Veterinary Sciences*, *6*(2), 75-80. https://doi.org/10.17582/journal.aavs/2018/6.2.75.80

Mettenleiter, T. C. (2000). Aujeszky's disease (pseudorabies) virus: The virus and molecular pathogenesis-state of the art, June 1999. *Veterinary Research*, *31*(1), 99-115. https://doi.org/10.1051/vetres:2000110

Mettenleiter, T. C. (2020). Aujeszky's disease and the development of the marker/DIVA vaccination concept. *Pathogens*, *9*(7), 563. https://doi.org/10.3390/pathogens9070563

Müller, T., Hahn, E. C., Tottewitz, F., Kramer, M., Klupp, B. G., Mettenleiter, T. C., & Freuling, C. (2011). Pseudorabies virus in wild swine: A global perspective. *Archives of Virology*, *156*, 1691-1705. https://doi.org/10.1007/s00705-011-1080-2

Nauwynck, H. J. (1997). Functional aspects of Aujeszky's disease (pseudorabies) viral proteins with relation to invasion, virulence and immunogenicity. *Veterinary Microbiology*, *55*(1-4), 3-11. https://doi.org/10.1016/s0378-1135(96)01299-0

Pomeranz, L. E., Reynolds, A. E., & Hengartner, C. J. (2005). Molecular biology of pseudorabies virus: Impact on neurovirology and veterinary medicine. *Microbiology and Molecular Biology Reviews*, *69*(3), 462-500. https://doi.org/10.1128/MMBR.69.3.462-500.2005

Siegel, A. M., & Weigel, R. M. (1999). Herd factors affecting the selection and success of intervention strategies in the program for eradication of pseudorabies (Aujeszky's disease) virus from Illinois swine farms. *Preventive Veterinary Medicine*, *40*(3-4), 243–259. https://doi.org/10.1016/s0167-5877(99)00026-4

Song, C., Gao, L., Bai, W., Zha, X., Yin, G., & Shu, X. (2017). Molecular epidemiology of pseudorabies virus in Yunnan and the sequence analysis of its gD gene. *Virus Genes*, *53*, 392-399. https://doi.org/10.1007/s11262-017-1429-z

Stegeman, A. (1995). Pseudorabies virus eradication by area-wide vaccination is feasible. *Veterinary Quarterly*, *17*(4), 150–156. https://doi.org/10.1080/01652176.1995.9694556

Sun, Y., Luo, Y., Wang, C.-H., Yuan, J., Li, N., Song, K., & Qiu, H.-J. (2016). Control of swine pseudorabies in China: Opportunities and limitations. *Veterinary Microbiology*, *183*, 119-124. https://doi.org/10.1016/j.vetmic.2015.12.008

Szczotka-Bochniarz, A., Lipowski, A., Kycko, A., Sell, B., Ziółkowski, M., & Małek, B. (2016). Wild boar offal as a probable source of Aujeszky's disease virus for hunting dogs in Poland. *Journal*

*of Veterinary Research*, *60*(3), 233-238. https://doi.org/10.1515/jvetres-2016-0035

Too, H. L. (1997). *Pig diseases in Malaysia*. Universiti Putra Malaysia Press.

Ukhovskyi, V. V., Korniienko, L. Y., Pyskun, A. V., Chechet, O. M., Drozhzhe, Z. M., Polupan, I. M., Aliekseieva, G. B., Moroz, O. A., & Romanov, O. M. (2022). Spread of Aujeszky's disease among wild boars and domestic pigs in Ukraine. *Regulatory Mechanisms in Biosystems*, *13*(1), 46-52. https://doi.org/10.15421/022206

Wang, Y. B., Li, Y. H., Li, Q. M., Xie, W. T., Guo, C. L., Guo, J. Q., Deng, R. G., & Zhang, G. P. (2019). Development of a blocking immunoperoxidase monolayer assay for differentiation between pseudorabies virus-infected and vaccinated animals. *Polish Journal of Veterinary Sciences*, *22*(4), 717-723. https://doi.org/10.24425/pjvs.2019.129985

van Oirschot, J. T., Gielkens, A. L. J., Moormann, R. J. M., & Berns, A. J. M. (1990). Marker vaccines, virus protein-specific antibody assays and the control of Aujeszky's disease. *Veterinary Microbiology*, *23*(1-4), 85–101. https://doi.org/10.1016/0378-1135(90)90139-m

Yang, H., Han, H., Wang, H., Cui, Y., Liu, H., & Ding, S. (2019). A case of human viral encephalitis caused by pseudorabies virus infection in China. *Frontiers in Neurology*, *10*, 534. https://doi.org/10.3389/fneur.2019.00534

Zheng, H.-H., Jin, Y., Hou, C.-Y., Li, X.-S., Zhao, L., Wang, Z.-Y., & Chen, H.-Y. (2021). Seroprevalence investigation and genetic analysis of pseudorabies virus within pig populations in Henan province of China during 2018-2019. *Infection, Genetics and Evolution*, *92*, 104835. https://doi.org/10.1016/j.meegid.2021.104835

Zuckermann, F. A. (2000). Aujeszky's disease virus: Opportunities and challenges. *Veterinary Research*, *31*(1), 121-131. https://doi.org/10.1051/vetres:2000111

Pertanika J. Trop. Agric. Sci. 47 (4): 1121 - 1137 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Insecticidal Potential of *Ocimum basilicum* Leaves: Metabolite Distribution in Different Leaf Tissues

**Nadya Sofia Siti Sa'adah, Nina Mutiara Calvaryni, Sukirno Sukirno, Laurentius Hartanto Nugroho and Tri Rini Nuringtyas***

*Faculty of Biology, Universitas Gadjah Mada, Jln. Teknika Selatan, Sekip Utara, Sleman 55281, Special Region of Yogyakarta, Indonesia*

## ABSTRACT

Leaves serve as essential plant organs that facilitate photosynthesis and consist of several layers, such as the mesophyll and epidermis, each of which possesses unique metabolite compositions. These metabolites play a role in the plant's defensive system against insects. For instance, the leaves of *Ocimum basilicum* L. (basil) possess biocidal properties against a variety of insects. Although the insecticidal properties of these leaves have been well documented, the distribution studies on the leaf metabolites are inadequate. Thus, this study examined the metabolite profiles of the two leaf layers, epidermis and mesophylls. The separation of epidermis and mesophyll extracts was accomplished using whetstone powder, followed by gas chromatography-mass spectrometry to analyze the obtained metabolite profiles. The leaf trichomes were examined by scanning electron microscopy. Certain chemicals were only detectable within the epidermal or mesophyll tissues. For example, tricosane (16.37%) and geraniol (7.88%) were exclusively detected in the epidermis, whereas limonene oxide (1.26%) and α-humulene (1.04%) were only detected in the mesophyll. Furthermore, certain components were found in higher quantities in the epidermis and mesophyll layers, whereas others were more prevalent in the opposite layer. Our findings relevant to the trichome types, specifically glandular and non-glandular trichomes, indicated that both play a role in the initial defenses against herbivorous insects. This study offers significant insights into the chemicals that serve as plant defenses in basil leaf tissue and trichomes. Future studies on the distribution of chemical compounds in different leaf tissues can provide further

ARTICLE INFO

*Article history:*
Received: 10 January 2024
Accepted: 05 March 2024
Published: 27 September 2024

DOI: https://doi.org/10.47836/pjtas.47.4.05

*E-mail addresses*:
nadya.sofia88@mail.ugm.ac.id (Nadya Sofia Siti Sa'adah)
Ncalvaryni@gmail.com (Nina Mutiara Calvaryni)
sukirnobiougm@ugm.ac.id (Sukirno Sukirno)
hartantonugroho2005@ugm.ac.id (Laurentius Hartanto Nugroho)
tririni@ugm.ac.id (Tri Rini Nuringtyas)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

insights into the mechanisms of plant-insect interaction and facilitate the development of strategies for identifying compounds that play a role in defense.

*Keywords*: Basil, epidermis, localization, gas chromatography-mass spectrometry, metabolite profile

## INTRODUCTION

Plants synthesize a wide array of chemical molecules and metabolites, including both primary and secondary metabolites. Primary metabolites are crucial in fundamental biological activities, including growth, cell division, photosynthesis, and respiration. In contrast, secondary metabolites interact between plants and their surrounding environment. Secondary metabolites are crucial in plant defense against herbivores and pathogens (Aharoni & Galili, 2011; Cavalier-Smith, 2007; Chaudhary et al., 2018; Erb & Kliebenstein, 2020). However, their structural diversity and accumulation characteristics in specific plant compartments remain poorly understood (Hadacek et al., 2011). Thus, specific secondary metabolites and their accumulation may play distinct roles against diverse herbivores (Berenbaum & Feeny, 1981; Nuringtyas et al., 2012).

Herbivorous insects attack plants by eating plant parts, selecting specific plant organs such as leaves, stems and roots, or only eating specific plant tissues (War et al., 2018). It raises the question about the effect of the way an herbivorous insect eats plants on the quality and quantity of mechanical damage to plant tissues. Leaf defoliators, such as caterpillars (Lepidoptera), cause tissue damage by chewing, cutting, and tearing. Leaf miners feed on soft plant parts between epidermal cells or mesophyll tissue layers in the leaves. Piercing/sucking insects such as thrips (Thysanoptera) have tube-like structures that suck fluid from lateral cells and the epidermis. Phloem-suckers such as aphids (Hemiptera) possess stylets that penetrate cells all the way into the phloem (Chaudhary et al., 2018; Fürstenberg-Hägg et al., 2013). When chewing insects attack plants, volatile organic compounds are released, attracting herbivorous insect enemies to reduce herbivorous insect numbers (Fürstenberg-Hägg et al., 2013). Therefore, chemical compounds and their composition in different organs and cell types are expressed differently to optimize plant defenses (Martin et al., 2001; Nuringtyas et al., 2012).

The *Ocimum* genus possesses abundant and diverse secondary metabolites, including terpenoids and phenolics, which may be involved in plant defenses. However, neither the species' defense mechanisms nor secondary metabolite roles have been well characterized, although the insecticidal activity of plant leaves against several insects has been reported. One of the *Ocimum* genus species reported to have insecticidal activity is *Ocimum kilimandscharicus*. This species reportedly possesses rich secondary metabolites such as eucalyptol, camphor, limonene, germacrene D, and β-caryophyllene (Singh et al., 2014). Another investigation was conducted to determine the metabolite profile of *Ocimum gratissimum* essential

oil (EO). The findings revealed that thymol and *p*-cymene predominate EO, whereas carvacrol and thymol are predominant in the ethanolic and aqueous extracts, respectively, along with shikimic acid and rosmarinic acid (Benelli et al., 2019). In addition, the EO of *O. basilicum* possesses noteworthy biological activity against insect pests. Chromatographic analysis of the oil revealed the presence of estragole and linalool (Boulamtat et al., 2021; da Silva Moura et al., 2020), methyl cinnamate, eugenol, 1,8-cineole, α-cadinol, α-bergamotene (Chaaban et al., 2019), and methyl eugenol (Govindarajan et al., 2013). Nevertheless, there has been a lack of research on the distribution of these metabolites within leaf tissues. Investigating the metabolite profile via mass spectrometry (MS) and its distribution in leaf tissues can help identify potential biopesticides and their mechanism of protecting plants from insects.

*Ocimum basilicum* exerts biocide activity against different herbivorous insects, possibly caused by glandular and non-glandular trichomes in the epidermis. Trichomes are important tissues that support plant defense systems; they facilitate plant resistance against herbivores via physical and chemical deterrents (Fürstenberg-Hägg et al., 2013). In addition to its role as a biopesticide, studies on *O. basilicum* have reported its role in supporting health. For instance, *O. basilicum* possesses antioxidant properties (Srivastava et al., 2016), therapeutic potential (Bensaid et al., 2022), and anti-acetylcholinesterase activity (Farag et al., 2016).

The carborundum abrasion (CA) method has been extensively employed for abrading the epidermis and mesophyll. Murata and De Luca (2005) implemented this method to collect epidermis samples to investigate alkaloid metabolites, enzyme activity, and gene expression levels. In addition, this technique allowed mRNA extraction from the leaf epidermis to assess the spatial distribution of indole alkaloid biosynthesis (Murata et al., 2008). According to Nuringtyas et al. (2012), the differences in the metabolic profiles were investigated between the epidermal and mesophyll tissues of *Jacobea vulgaris* and *Jacobea aquatica* using CA. A similar approach was employed to investigate the accumulation of putative photoprotective group chemicals (Ilmiah et al., 2018). This study involved the optimization of CA to achieve the full removal of the leaf epidermis using whetstone powder. The fundamental idea behind this approach is to use silicon carbide powder to abrade the epidermal layer.

The current research on plant defense chemicals detected in basil leaf tissues is limited, and no studies exist that distinguish compounds in different tissue layers of basil leaves. In fact, the existing research has focused only on the content of secondary metabolites in basil. For instance, Chaaban et al. (2019), da Silva Moura et al. (2020), and Govindarajan et al. (2013) only evaluated the activity of crude basil extracts against insect pests. The present study is the first on defense chemicals found in different tissue layers of basil leaves. In this work,

the compounds in basil leaf tissues were separated using whetstone powder and the component distribution was analyzed. The current investigation serves as the first attempt to gain a deeper understanding of the underlying mechanisms of plant defense in basil leaf tissues by examining metabolite and trichrome profiles.

## MATERIALS AND METHODS

### Plant Material

In June 2022, fresh basil leaf samples were collected from Sengi Village, Magelang, Central Java, Indonesia (7°31'31.5''S, 110°21'47.0''E). The leaves were removed from plants that had just entered the flowering phase, which is high in EO production (Marotti et al., 1996). Basil plant species were authenticated at the Plant Systematic Laboratory, Faculty of Biology, Universitas Gadjah Mada, Indonesia.

### Epidermis and Mesophyll Extraction

Epidermal extraction was performed using a modified whetstone powder approach from Ilmiah et al. (2018). Briefly, the abaxial and adaxial epidermis layers were lightly rubbed six times using whetstone powder. The abraded leaves were then placed into a 50-ml conical tube with *n*-hexane solvent and vortexed for 1 min to separate the powder from the leaves. The extract was then filtered and placed on a porcelain dish to allow solvent evaporation. The abraded leaves were defined as mesophyll, removed from the conical tube, and ground in a blender. The fine-ground leaves were then extracted by maceration in *n*-hexane solvent for 24 hr at the leaf: solvent ratio of 1:5. After 24 hr, the mesophyll extract was filtered and poured into a porcelain dish to allow solvent evaporation. The samples were then stored at 4°C until further requirement.

### Microscopic Analysis

Microscopic observations of the leaf surfaces before and after whetstone powder extraction were performed to compare the epidermis layers. Before microscopy, longitudinal leaf sections were prepared using a sliding microtome (REICHERT Nr. 338 262, Austria). Subsequently, the specimens were observed under light microscopy (BOECO, BM-180, Germany) at 100× magnification.

### Gas Chromatography-Mass Spectrometry (GC–MS) Metabolite Analysis of the Epidermis and Mesophyll

GC-MS was conducted at the Department of Organic Chemistry, Universitas Gadjah Mada, using (GCMS-QP2010S, Shimadzu Corporation, Japan) with single-quadrupole MS attached to a DB-5MS capillary column operated in the electronic ionization mode at 70 eV. The dried extract was diluted again with the solvent. Helium was used as the carrier gas, and the analyses were conducted using the splitless method with the following settings: column oven temperature = 60°C, sampling time = 1.00 min, flow control mode: pressure, injection temperature = 300°C, pressure = 16.5 kPa, total flow = 30.5 ml/min, column flow = 0.55 ml/min, linear

velocity = 27.1 cm/s, and purge flow = 3.0 ml/min. Finally, the chromatogram peaks were analyzed.

### Data Analysis

GC-MS chromatogram data were cleaned by eliminating the MS values that resembled impurities. The MS splitting patterns were compared with the MS information in PubChem and KNApSAck-3D databases (http://kanaya.naist.jp/knapsack esp top. html) to validate and identify compounds. The mass spectral interpretation was based on compounds with similarity indices >86% (Dahibhate et al., 2022). The compounds were assembled into a heatmap plot using GraphPad Prism 9 for better representation. A higher value set percentage area was set for 15, and all values >15 were colored as the highest concentration.

### Scanning Electron Microscopy (SEM) Analysis

SEM analysis was conducted at the Integrated Research and Testing Laboratory, Universitas Gadjah Mada. The samples were sliced into ± 5 × 7-mm sections and affixed to carbon tape above the specimen holder. The samples were then inserted into the auto coater. A vacuum coater machine applied a pressure of ±3.2 Pa, followed by coating with a layer of gold. Subsequently, the samples were placed into the SEM and subjected to vacuum, and electrons fired the sample with a certain level probe. The upper and lower epidermal surface observations were then recorded.

## RESULTS

### Epidermis and Mesophyll Abrasion

Epidermal abrasion using whetstone powder is depicted in Figures 1a and 1b. Observations were made using cross-sectional preparations under microscopy. This approach successfully eroded the epidermis by lightly rubbing the surface six times. Abrasion treatment with whetstone powder could successfully erode basil leaves' adaxial and abaxial epidermis layers.

### Compound Identification by GC–MS

Epidermis extract GC–MS chromatograms displayed 28 peaks (Figure 2a), whereas the mesophyll extracts showed 46 peaks with varying percentages (Figure 2b). Different secondary metabolites were

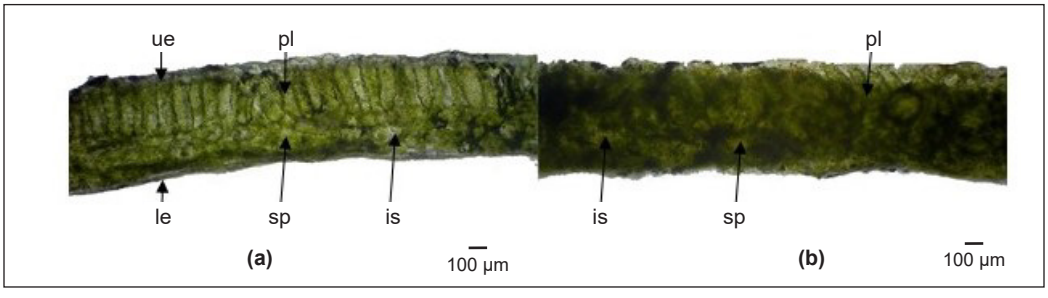



*Figure 1.* Microscopic comparison of *Ocimum basilicum* leaf cross-sections before (a) and after (b) whetstone powder abrasion: the upper epidermis (ue), palisade (pl), sponge (sp), intercellular space (is), and lower epidermis (le)

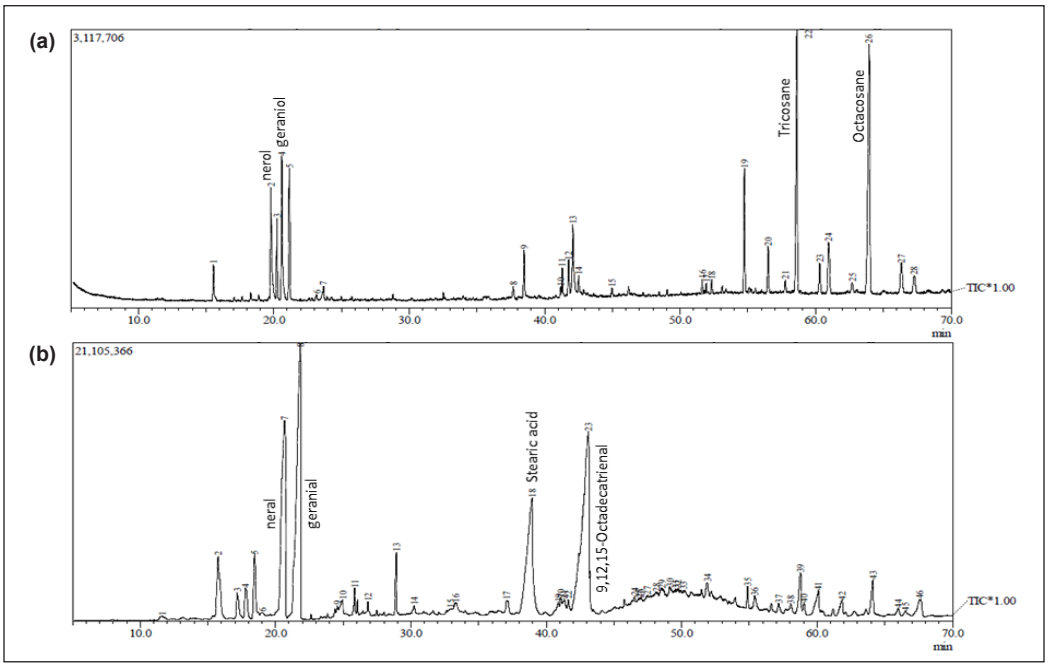



*Figure 2*. Gas chromatography-mass spectrometry chromatograms of bioactive components from basil (*Ocimum basilicum*) leaf: (a) epidermis and (b) mesophyll extracts, respectively

identified using chromatograms' mass spectra and compound detection (Table 1). A typically different chromatogram of the different tissues was observed immediately, wherein the epidermis showed significant signals at retention time (RT) of 58.62 and 65.95 min, corresponding to tricosane and octacosane. The mesophyll metabolite profiles had significant signals at RT 20.23, 21.15, 42.49, and 43.10 min, which were identified as neral, geranial, stearic acid, and 9,12,15-octadecatrienal, respectively.

A heatmap of the basil leaf epidermis and mesophyll compounds was assembled to visualize metabolite distribution (based on the compound percentage areas; Figure 3). Dark blue indicates a high compound concentration, while white indicates low concentrations. Several compounds, including tricosane, octacosane, nerol, and geraniol, were detected at high concentrations in the epidermal extracts. In contrast, the mesophyll extracts detected neral, geranial, stearic acid, and 9,12,15 octadecatrienal at high concentrations.

## Trichome Analysis Using SEM

Trichome morphology and distribution were compared on the adaxial and abaxial leaf surfaces (Figure 4). SEM revealed that the basil leaves had two trichome types, glandular and non-glandular (Figures 4a and b), which were nonuniformly distributed on the adaxial and abaxial epidermis surfaces. The adaxial sections showed more non-glandular trichomes, whereas the abaxial sections showed more glandular trichomes (Figure 4d).

Table 1
*Bioactive compounds in the basil leaf epidermis and mesophyll extracts*

| No. | RT | Compounds | Groups | MF | MW | Area (%) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Epidermis | Mesophyll |
| 1. | 15.56 | 1,6-Octadien-3-ol, 3,7-dimethyl- (linalool) | Monoterpene | $C_{10}H_{18}O$ | 154 | 1.82 | 3.27 |
| 2. | 17.79 | Limonene oxide | Monoterpene | $C_{10}H_{16}O$ | 152 | - | 1.26 |
| 3. | 18.45 | Limonene epoxide | Monoterpene | $C_{10}H_{16}O$ | 152 | - | 2.56 |
| 4. | 19.79 | 2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)- (nerol) | Monoterpene | $C_{10}H_{18}O$ | 154 | **7.25** | 1.08 |
| 5. | 20.23 | 2,6-Octadienal, 3,7-dimethyl-, (Z)- (neral) | Monoterpene | $C_{10}H_{16}O$ | 152 | 3.38 | **14.64** |
| 6. | 20.59 | 2,6-Octadien-1-ol, 3,7-dimethyl-, (E)- (geraniol) | Monoterpene | $C_{10}H_{18}O$ | 154 | **7.88** | - |
| 7. | 21.15 | 2,6-Octadienal, 3,7-dimethyl-, (E)- (geranial) | Monoterpene | $C_{10}H_{16}O$ | 152 | 4.96 | **18.13** |
| 8. | 24.96 | 4-Methyl-3-penten-1-ol | Homoallylic alcohol | $C_6H_{12}O$ | 100 | - | 1.00 |
| 9. | 28.90 | alpha.-Humulene | Sesquiterpene | $C_{15}H_{24}$ | 204 | - | 1.04 |
| 10. | 38.49 | Hexadecanoic acid (palmitic acid) | Fatty acid | $C_{16}H_{32}O_2$ | 256 | 2.26 | - |
| 11. | 41.29 | 11-Octadecenoic acid, methyl ester | Fatty acid | $C_{19}H_{36}O_2$ | 296 | 1.18 | - |
| 12. | 42.10 | 9-Octadecenal | Fatty aldehyde | $C_{18}H_{34}O$ | 266 | 5.03 | - |
| 13. | 42.49 | Stearic acid | Fatty acid | $C_{18}H_{36}O_2$ | 284 | 1.23 | **12.00** |
| 14. | 43.10 | 9,12,15-Octadecatrienal | Linolenyl aldehyde | $C_{18}H_{30}O$ | 262 | - | **21.52** |
| 15. | 48.55 | 1-Octanol, 2-butyl- | Fatty alcohol | $C_{12}H_{26}O$ | 186 | - | 1.14 |
| 16. | 54.74 | Dotriacontane | Alkanes | $C_{32}H_{66}$ | 450 | 5.72 | - |
| 17. | 56.52 | Pentacosane | Alkanes | $C_{22}H_{52}$ | 352 | 2.29 | - |
| 18. | 58.62 | Tricosane | Alkanes | $C_{23}H_{48}$ | 324 | **16.37** | - |
| 19. | 58.78 | Tetradecane | Alkanes | $C_{14}H_{30}$ | 198 | - | 1.53 |
| 20. | 60.31 | Eicosane | Alkanes | $C_{20}H_{42}$ | 282 | 1.96 | - |
| 21. | 60.98 | Nonacosane | Alkanes | $C_{29}H_{60}$ | 408 | 3.60 | - |
| 22. | 63.95 | Octacosane | Alkanes | $C_{28}H_{58}$ | 394 | **22.43** | 1.35 |
| 23. | 66.34 | Hexatriacontane | Alkanes | $C_{36}H_{74}$ | 507 | 2.87 | - |
| 24. | 67.28 | Eicosane, 2-methyl- | Alkanes | $C_{21}H_{44}$ | 296 | 1.74 | - |

*Note*. Bold numbers indicate the major compounds in the extracts; RT = Retention time; MF = Molecular formula; MW = Molecular weight

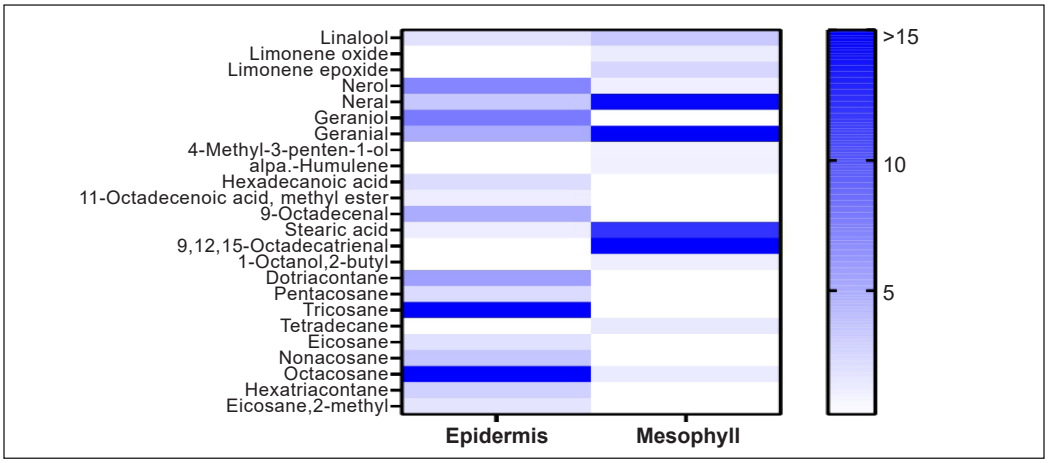



*Figure 3*. Heatmap showing epidermis and mesophyll basil leaf extracts based on gas chromatography-mass spectrometry analyses

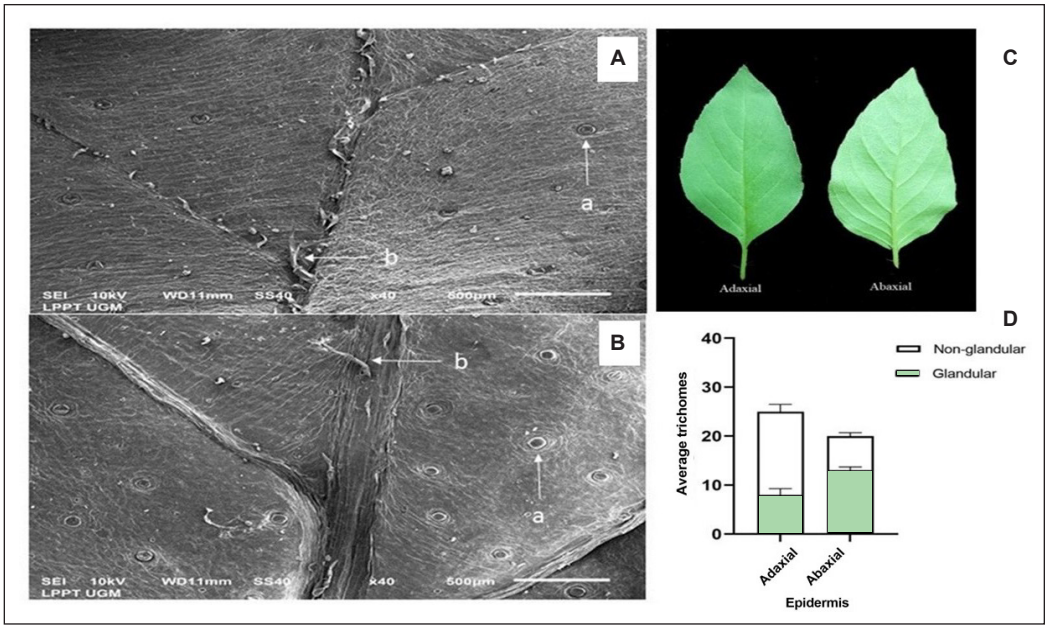



*Figure 4*. *Ocimum basilicum* leaves. Trichomes on adaxial (A) and abaxial (B) leaves (scanning electron microscopy): glandular trichomes (a) and non-glandular trichomes (b). The physical appearance of basil leaves (C) and the average numbers of adaxial and abaxial trichomes (D), respectively

## DISCUSSION

Until recently, leaf metabolite profiling was largely conducted without considering the secondary metabolite distribution across different tissues. Different insect types must be considered when examining secondary metabolites as defense chemicals. For instance, sucking insects are exposed to different chemicals when compared with chewing insects, which consume the entire

plant's leaves (Nuringtyas et al., 2012). In our study, the epidermis and mesophyll layers were used to determine the location of chemical compounds or chemical collections. A comparative investigation of metabolites in these layers may provide insights into the metabolite accumulation in different tissues and their roles as defense chemicals against herbivore insects and pathogens.

The CA method is the most common and straightforward method of isolating the epidermis (Ilmiah et al., 2018; Murata et al., 2008; Nuringtyas et al., 2012). Ilmiah et al. (2018) used whetstone powder to replace CA because it could not erode the *Sonneratia caseolaris* (L.) leaf epidermis. Several factors can affect tissue separation, including the leaf anatomy, cell wall thickness, and the pressure applied to leaves (Ilmiah et al., 2018). The basil leaves epidermis (upper and lower) and mesophyll tissues were effectively separated using whetstone powder, as shown in Figure 1. When compared with CA, whetstone powder offers several advantages, such as its ample availability and lesser cost. Therefore, whetstone powder was used to separate and isolate plant tissues in this study.

Lamiaceae plants are distinguished by their ability to synthesize the most volatile terpenes in glandular trichomes. These organs possess a high concentration of volatile compounds, enhancing their botanical attributes as aromatic plants (de Sena Filho et al., 2023). EO derived from *Ocimum*, a member of the Lamiaceae family, is commonly obtained from hydrodistillation. It has been documented to possess various properties, such as antibacterial and biopesticide activities (da Silva Moura et al., 2020; Sneha et al., 2022). The selection of maceration with n-hexane as the solvent in this study was based on a previous study conducted by Kayesth et al. (2018). Their study demonstrated the discovery of various chemical compounds with insecticidal properties. This finding aligns with the objective of the present study, which identifies a wider range of chemicals hypothesized to contribute to plant defense mechanisms.

In a comparison of compounds between the epidermis and mesophyll tissue extracts by GC–MS, several compounds were observed only in the epidermis or mesophyll tissues. In addition, other compounds were more abundant in the epidermis or mesophyll layers and *vice versa* (Table 1; Figure 3). Geraniol is a monoterpenoid alcohol that contains a mixture of two cis-trans isomers-geraniol and nerol (Chen & Viljoen, 2010). Both were sevenfold higher in the epidermis than in the mesophyll tissues (Table 1). Research has indicated the presence of geraniol as a rich component in EOs of plants such as *Cymbopogon* spp. and *Pelargonium graveolens* (Dangol et al., 2023; Džamić et al., 2014).

As common components of some EOs, geraniol and nerol have promising biological properties. Both possess strong antifungal activities against *Fusarium* species, such as *Fusarium verticillioides* (Brito et al., 2019) and *Fusarium graminearum* (Krzysko-Łupicka et al., 2019). Geraniol demonstrated

fumigant potency against common pests in stored products, *Sitophilus zeamais* (Coleoptera: Curculionidae) and *Lipocelis bostrychophila* (Psocodea: Liposcelididae) (Quan et al., 2018), along with *Tuta absoluta* (Lepidoptera: Gelechiidae), a major tomato plant pest (Rahmani & Azimi, 2020). Thus, geraniol and nerol in epidermis layers may act as first-line defense mechanisms against pathogens or pests.

Citral is a mixture of monoterpenoid aldehydes, predominantly neral and geranial, and has higher area percentages in the mesophyll tissues (Table 1). The percentage area of neral in mesophyll was fivefold higher than in the epidermis tissues. In addition, the percentage area for geranial cells increased fourfold in the mesophyll when compared with other compounds. High citral accumulation in the mesophyll was also reported in mesophyll lemongrass (*Cymbopogon flexuasus*), determined using a histochemical approach (Lewinsohn et al., 1998). Furthermore, Dancewicz et al. (2020) showed that citral is a strong repellent and functioned as a pre-and ingestive probing deterrent to *Myzus persicae* (Hemiptera: Aphididae). Citral was also found responsible for the insecticidal activity of lemongrass extracts against *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (de Oliveira et al., 2018).

Citral and geraniol are often investigated with respect to their biopesticide and pharmaceutical characteristics. In this study, the accumulation in different tissues observed for geraniol and nerol when compared with the citral component, that is, geranial and neral, is interesting to observe. In terms of biosynthesis, these monoterpenes are derived from the same pathways, either through the cytoplasmic mevalonate pathway or the plastid 2-C-methyl-d-erythritol 4-phosphate pathway (Lewinsohn et al., 1998). However, specific metabolite accumulation has been associated with specialized secretory structures inside leaves, such as glandular trichomes, resin ducts, and glandular epidermis (Luthra et al., 2017). Neral and geranial citral components are obtained as nerols and geraniol oxidation products (Kanehisa & Goto, 2000).

Distribution differences were observed in comparison of GC–MS-identified compounds in the epidermis and mesophyll tissues (Table 1). Compounds identified only in the epidermis tissues with significant area percentages include tricosane and octacosane. Concurrently, compounds identified only in mesophyll tissues with significant area percentages include stearic acid and 9,12,15-octadecatrienal. The higher stearic acid levels observed in this study are consistent with the report of Lessire and Stumpf (1983), who reported that palmitic and stearic acid distributions were localized. Palmitic acid is a major product of epidermis cells, whereas stearic and palmitic acids are in parenchymal cells.

Tricosane is an oil component in plants and insects. It is extensively distributed and reportedly occurs in more than 50 plant species. However, the role of this compound in plant resistance remains unclear. Al-Maawali et al. (2021) reported that tricosane is an antifungal compound that acted against

tomato rot caused by *Alternaria alternata*. In insects, tricosane is a common female pheromone. In addition, some insects may exploit these compounds to assist hormone formation. Interestingly, this compound was also found in insect frass and functioned as an oviposition deterrent for the potato tubeworm moth *Phthorimaea operculella* (Lepidoptera: Gelechiidae) (Zhang et al., 2019).

Other specific compounds in tissue extracts were also identified, and their biological activities have been previously reported. In mesophyll tissue extracts, limonene oxide and α-humulene were identified. Limonene oxide was reported as a repellent for the tomato leaf miner *Tuta absoluta* (Miano et al., 2022). The α-humulene bioavailability in plants may indicate plant defense responses. Critically, *Spodoptera litura* (Lepidoptera: Noctuidae) reacted to this compound and may have signaling roles when locating hosts (Jönsson & Anderson, 1999).

In our study, 9,12,15-octadecatrienal was the most abundant compound in the mesophyll and reportedly regulated *Plutella xylostella* L. (diamond moth) (Lepidoptera: Plutellidae) behavior on pakchoi leaves (Zhandi et al., 2021). Thus, 9,12,15-octadecatrienal could be used to develop lead compounds for the green control of *P. xylostella* (Zhandi et al., 2021). *Plutella xylostella* attacks many vegetables, especially the Brassicaceae family, and targets young and old leaves during the larval stages.

The presence of other phenylpropenes was not detected because of several factors. Research conducted by Ahmed et al. (2019) explained that the chemical composition of *O. basilicum* can change according to the geographical location. In addition, research conducted by Dmitruk et al. (2019) also mentioned that the composition of EOs and their quantitative content depend on the part of the plant used and the plant's growth stage during the extraction.

Two major glandular and non-glandular trichome types were observed on basil leaves. Physical and chemical deterrents showcase first-line plant defense roles against herbivorous insects (Peiffer et al., 2009; Tian et al., 2012; Wang et al., 2021). Kariyat et al. (2017) demonstrated that non-glandular *Solanum* spp. trichomes functioned as mechanical post-ingestive defense mechanisms by harming the peritrophic matrix in caterpillars. More non-glandular trichomes were observed on basil adaxial leaf surfaces than on glandular trichomes, but the opposite was observed on abaxial leaf surfaces. Reportedly, jasmonic acid may account for this variation in trichrome synthesis (Tian et al., 2012; Traw & Bergelson, 2003; van Schie et al., 2007). Moreover, several studies demonstrated that glandular and non-glandular trichome density levels on leaves were possibly influenced by environmental factors, such as water availability and herbivore presence (Gonzáles et al., 2008; Rautio et al., 2002; Sá et al., 2016; Sletvold et al., 2010).

When defending against herbivorous insects, glandular trichomes also contain chemicals such as volatile terpenes (Iijima et al., 2004; Peiffer et al., 2009), which

release alkaloids, repellents, and poisonous compounds (Wang et al., 2021). Gang et al. (2002) reported that enzymes involved in phenylpropene production (such as chavicol, eugenol, and derivatives) were localized in the trichomes of basil leaves. Xie et al. (2008) also characterized the terpenoid metabolism processes in basil leaf trichomes.

## CONCLUSION

In this study, the epidermal and mesophyll tissue metabolite profiles in basil leaves were successfully distinguished using the CA technique. This technique allows the identification of several chemicals with varying concentrations in distinct layers. The localization of promising compounds, geraniol and citral, respectively, in the epidermis and mesophyll layer of *O. basilicum*, leaves implies that different tissues play different roles in plant defense against pests. Moreover, distinct trichrome distribution patterns were observed on basil leaves' upper and lower epidermal surfaces. The results of our study offer more knowledge regarding the mechanisms of plant defense *in O. basilicum* leaf tissues.

## ACKNOWLEDGMENTS

The authors would like to acknowledge the Directorate of Research and Community Service, Deputy for Strengthening Research and Development, Ministry of Research, Technology/National Research and Innovation Agency of the Republic of Indonesia in the Pendidikan Magister menuju Doktor untuk Sarjana Unggul (PMDSU) program with research grant 2021 contract No. 4453/UN1/DITLIT/DIT-LIT/PT/2021.

## REFERENCES

Aharoni, A., & Galili, G. (2011). Metabolic engineering of the plant primary-secondary metabolism interface. *Current Opinion in Biotechnology*, *22*(2), 239–244. https://doi.org/10.1016/j.copbio.2010.11.004

Ahmed, A. S., Fanokh, A. K. M., & Mahdi, M. A. (2019). Phytochemical identification and antioxidant study of essential oil constituents of *Ocimum basilicum* L. growing in Iraq. *Pharmacognosy Journal*, *11*(4), 724–729. https://doi.org/10.5530/pj.2019.11.115

Al-Maawali, S. S., Al-Sadi, A. M., Alsheriqi, S. A. K., Al-Sabahi, J. N., & Velazhahan, R. (2021). The potential of antagonistic yeasts and bacteria from tomato phyllosphere and fructoplane in the control of *Alternaria* fruit rot of tomato. *All Life*, *14*(1), 34-48. https://doi.org/10.1080/26895293.2020.1858975

Benelli, G., Pavela, R., Maggi, F., Wandjou, J. G. N., Fofie, N. G. B. Y., Koné-Bamba, D., Sagratini, G., Vittori, S., & Caprioli, G. (2019). Insecticidal activity of the essential oil and polar extracts from *Ocimum gratissimum* grown in Ivory Coast: Efficacy on insect pests and vectors and impact on non-target species. *Industrial Crops and Products*, *132*, 377-385. https://doi.org/10.1016/j.indcrop.2019.02.047

Bensaid, A., Boudard, F., Servent, A., Morel, S., Portet, K., Guzman, C., Vitou, M., Bichon, F., & Poucheret, P. (2022). Differential nutrition-health properties of *Ocimum basilicum* leaf and stem extracts. *Foods*, *11*(12), 1699. https://doi.org/10.3390/foods11121699

Berenbaum, M., & Feeny, P. (1981). Toxicity of angular furanocoumarins to swallowtail

butterflies: Escalation in a coevolutionary arms race? *Science*, *212*(4497), 927–929. https://doi.org/10.1126/science.212.4497.927

Boulamtat, R., Mesfioui, A., El-Fakhouri, K., Oubayoucef, A., Sabraoui, A., Aasfar, A., & El-Bouhssini, M. (2021). Chemical composition, and insecticidal activities of four plant essential oils from Morocco against larvae of *Helicoverpa armigera* (Hub.) under field and laboratory conditions. *Crop Protection*, *144*, 105607. https://doi.org/10.1016/j.cropro.2021.105607

Brito, V. D., Achimón, F., Dambolena, J. S., Pizzolitto, R. P., & Zygadlo, J. A. (2019). Trans-2-hexen-1-ol as a tool for the control of *Fusarium verticillioides* in stored maize grains. *Journal of Stored Products Research*, *82*, 123–130. https://doi.org/10.1016/j.jspr.2019.05.002

Cavalier-Smith, T. (2007). Origins of secondary metabolism. In D. J. Chadwick & J. Whelan (Eds.), *Ciba Foundation Symposium 171 - Secondary Metabolites: Their Function and Evolution* (pp. 64-87). Ciba Foundation. https://doi.org/10.1002/9780470514344.ch5

Chaaban, S. B., Hamdi, S. H., Mahjoubi, K., & Jemâa, J. M. B. (2019). Composition and insecticidal activity of essential oil from *Ruta graveolens*, *Mentha pulegium* and *Ocimum basilicum* against *Ectomyelois ceratoniae* Zeller and *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). *Journal of Plant Diseases and Protection*, *126*, 237-246. https://doi.org/10.1007/s41348-019-00218-8

Chaudhary, A., Bala, K., Thakur, S., Kamboj, R., & Dumra, N. (2018). Plant defenses against herbivorous insects: A review. *International Journal of Chemical Studies*, *6*(5), 681–688.

Chen, W., & Viljoen, A. M. (2010). Geraniol — A review of a commercially important fragrance material. *South African Journal of Botany*, *76*(4), 643–651. https://doi.org/10.1016/j.sajb.2010.05.008

da Silva Moura, E., D'Antonino Faroni, L. R., Heleno, F. F., Rodrigues, A. A. Z., Prates, L. H. F., & de Queiroz, M. E. L. R. (2020). Optimal extraction of *Ocimum basilicum* essential oil by association of ultrasound and hydrodistillation and its potential as a biopesticide against a major stored grains pest. *Molecules*, *25*(12), 2781. https://doi.org/10.3390/molecules25122781

Dahibhate, N. L., Dwivedi, P., & Kumar, K. (2022). GC–MS and UHPLC-HRMS based metabolite profiling of *Bruguiera gymnorhiza* reveals key bioactive compounds. *South African Journal of Botany*, *149*, 1044-1048. https://doi.org/10.1016/j.sajb.2022.02.004

Dancewicz, K., Szumny, A., Wawrzeńczyk, C., & Gabryś, B. (2020). Repellent and antifeedant activities of citral-derived lactones against the peach potato aphid. *International Journal of Molecular Sciences*, *21*(21), 8029. https://doi.org/10.3390/ijms21218029

Dangol, S., Poudel, D. K., Ojha, P. K., Maharjan, S., Poudel, A., Satyal, R., Rokaya, A., Timsina, S., Dosoky, N. S., Satyal, P., & Setzer, W. N. (2023). Essential oil composition analysis of *Cymbopogon* species from eastern Nepal by GC-MS and chiral GC-MS, and antimicrobial activity of some major compounds. *Molecules*, *28*(2), 543. https://doi.org/10.3390/molecules28020543

de Oliveira, E. R., Alves, D. S., Carvalho, G. A., de Oliveira, B. M. R. G., Aazza, S., & Bertolucci, S. K. V. (2018). Toxicity of *Cymbopogon flexuosus* essential oil and citral for *Spodoptera frugiperda*. *Ciência e Agrotecnologia*, *42*(4), 408–419. https://doi.org/10.1590/1413-70542018424013918

de Sena Filho, J. G., de Almeida, A. S., Pinto-Zevallos, D., Barreto, I. C., de Holanda Cavalcanti, S. C., Nunes, R., Teodoro, A. V., Xavier, H. S., Filho, J. M. B., Guan, L., Neves, A. L. A., & Duringer, J. M. (2023). From plant scent defense to biopesticide discovery: Evaluation of toxicity

and acetylcholinesterase docking properties for Lamiaceae monoterpenes. *Crop Protection*, *164*, 106126. https://doi.org/10.1016/j.cropro.2022.106126

Dmitruk, M., Sulborska, A., Żuraw, B., Stawiarz, E., & Weryszko-Chmielewska, E. (2019). Sites of secretion of bioactive compounds in leaves of *Dracocephalum moldavica* L.: Anatomical, histochemical, and essential oil study. *Brazilian Journal of Botany*, *42*, 701–715. https://doi.org/10.1007/s40415-019-00559-6

Džamić, A. M., Soković, M. D., Ristić, M. S., Grujić, S. M., Mileski, K. S., & Marin, P. D. (2014). Chemical composition, antifungal and antioxidant activity of *Pelargonium graveolens* essential oil. *Journal of Applied Pharmaceutical Science*, *4*(3), 1–5. https://doi.org/10.7324/JAPS.2014.40301

Erb, M., & Kliebenstein, D. J. (2020). Plant secondary metabolites as defenses, regulators, and primary metabolites: The blurred functional trichotomy. *Plant Physiology*, *184*(1), 39–52. https://doi.org/10.1104/PP.20.00433

Farag, M. A., Ezzat, S. M., Salama, M. M., & Tadros, M. G. (2016). Anti-acetylcholinesterase potential and metabolome classification of 4 *Ocimum* species as determined *via* UPLC/qTOF/MS and chemometric tools. *Journal of Pharmaceutical and Biomedical Analysis*, *125*, 292-302. https://doi.org/10.1016/j.jpba.2016.03.037

Fürstenberg-Hägg, J., Zagrobelny, M., & Bak, S. (2013). Plant defense against insect herbivores. *International Journal of Molecular Sciences*, *14*(5), 10242-10297. https://doi.org/10.3390/ijms140510242

Gang, D. R., Beuerle, T., Ullmann, P., Werck-Reichhart, D., & Pichersky, E. (2002). Differential production of meta hydroxylated phenylpropanoids in sweet basil peltate glandular trichomes and leaves is controlled by the activities of specific acyltransferases and hydroxylases. *Plant Physiology*, *130*(3), 1536–1544. https://doi.org/10.1104/pp.007146

Gonzáles, W. L., Negritto, M. A., Suárez, L. H., & Gianoli, E. (2008). Induction of glandular and non-glandular trichomes by damage in leaves of *Madia sativa* under contrasting water regimes. *Acta Oecologica*, *33*(1), 128-132. https://doi.org/10.1016/j.actao.2007.10.004

Govindarajan, M., Sivakumar, R., Rajeswary, M., & Yogalakshmi, K. (2013). Chemical composition and larvicidal activity of essential oil from *Ocimum basilicum* (L.) against *Culex tritaeniorhynchus*, *Aedes albopictus* and *Anopheles subpictus* (Diptera: Culicidae). *Experimental Parasitology*, *134*(1), 7-11. https://doi.org/10.1016/j.exppara.2013.01.018

Hadacek, F., Bachmann, G., Engelmeier, D., & Chobot, V. (2011). Hormesis and a chemical raison d'être for secondary plant metabolites. *Dose-Response*, *9*(1). https://doi.org/10.2203/dose-response.09-028.Hadacek

Iijima, Y., Gang, D. R., Fridman, E., Lewinsohn, E., & Pichersky, E. (2004). Characterization of geraniol synthase from the peltate glands of sweet basil. *Plant Physiology*, *134*(1), 370–379. https://doi.org/10.1104/pp.103.032946

Ilmiah, H. H., Nuringtyas, T. R., & Nugroho, L. H. (2018). Accumulation of potential photoprotective compound groups in mangrove (*Sonneratia caseolaris* (L.) Engler.) leaves. *Pharmacognosy Journal*, *10*(3), 576–580. https://doi.org/10.5530/pj.2018.3.94

Jönsson, M., & Anderson, P. (1999). Electrophysiological response to herbivore-induced host plant volatiles in the moth *Spodoptera littoralis*. *Physiological Entomology*, *24*(4), 377–385. https://doi.org/10.1046/j.1365-3032.1999.00154.x

Kanehisa, M., & Goto, S. (2000). KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Research*, *28*(1), 27–30.

Kariyat, R. R., Smith, J. D., Stephenson, A. G., De Moraes, C. M., & Mescher, M. C. (2017). Non-glandular trichomes of *Solanum carolinense* deter feeding by *Manduca sexta* caterpillars and cause damage to the gut peritrophic matrix. *Proceedings of the Royal Society B: Biological Sciences*, *284*, 20162323. https://doi.org/10.1098/rspb.2016.2323

Kayesth, S., Gupta, K. K., Kumar, S., & Shazad, M. (2018). Effects of *Ocimum sanctum* hexane extract on survival and development of *Dysdercus koenigii* Fabricius (Heteroptera: Pyrrhocoriedae). *Archives of Phytopathology and Plant Protection*, *51*(17–18), 993–1007. https://doi.org/10.1080/03235408.2018.1541148

Krzysko-Łupicka, T., Walkowiak, W., & Bialon, M. (2019). Comparison of the fungistatic activity of selected essential oils relative to *Fusarium graminearum* isolates. *Molecules*, *24*(2), 311. https://doi.org/10.3390/molecules24020311

Lessire, R., & Stumpf, P. K. (1983). Nature of the fatty acid synthetase systems in parenchymal and epidermal cells of *Allium porrum* L. leaves. *Plant Physiology*, *73*(3), 614-618. https://doi.org/10.1104/pp.73.3.614

Lewinsohn, E., Dudai, N., Tadmor, K., Katzir, I., Ravid, U., Putievsky, E., & Joels, D. M. (1998). Histochemical localization of citral accumulation in lemongrass leaves (*Cymbopogon citratus* (DC.) Stapf., Poaceae). *Annals of Botany*, *81*(1), 35–39. https://doi.org/10.1006/anbo.1997.0525

Luthra, R., Srivastava, A. K., & Ganjewala, D. (2017). Histochemical localisation of citral accumulating cite in lemongrass (*Cymbopogon flexuosus* Ness Ex. Steud) wats cultivar OD-19. *Asian Journal of Plant Science*, *6*, 419–422. https://doi.org/10.3923/ajps.2007.419.422

Marotti, M., Piccaglia, R., & Giovanelli, E. (1996). Differences in essential oil composition of Basil (*Ocimum basilicum* L.) Italian cultivars related to morphological characteristics. *Journal of Agricultural and Food Chemistry*, *44*(12), 3926–3929. https://doi.org/10.1021/jf9601067

Martin, C., Bhatt, K., & Baumann, K. (2001). Shaping in plant cells. *Current Opinion in Plant Biology*, *4*(6), 540–549. https://doi.org/10.1016/S1369-5266(00)00213-2

Miano, R. N., Ayelo, P. M., Musau, R., Hassanali, A., & Mohamed, S. A. (2022). Electroantennogram and machine learning reveal a volatile blend mediating avoidance behavior by *Tuta absoluta* females to a wild tomato plant. *Scientific Reports*, *12*, 8965. https://doi.org/10.1038/s41598-022-13125-0

Murata, J., & De Luca, V. (2005). Localization of tabersonine 16-hydroxylase and 16-OH tabersonine-16-*O*-methyltransferase to leaf epidermal cells defines them as a major site of precursor biosynthesis in the vindoline pathway in *Catharanthus roseus*. *The Plant Journal*, *44*(4), 581-594. https://doi.org/10.1111/j.1365-313X.2005.02557.x

Murata, J., Roepke, J., Gordon, H., & De Luca, V. (2008). The leaf epidermome of *Catharanthus roseu*s reveals its biochemical specialization. *Plant Cell*, *20*(3), 524–542. https://doi.org/10.1105/tpc.107.056630

Nuringtyas, T. R., Choi, Y. H., Verpoorte, R., Klinkhamer, P. G. L., & Leiss, K. A. (2012). Differential tissue distribution of metabolites in *Jacobaea vulgaris*, *Jacobaea aquatica* and their crosses. *Phytochemistry*, *78*, 89–97. https://doi.org/10.1016/j.phytochem.2012.03.011

Peiffer, M., Tooker, J. F., Luthe, D. S., & Felton, G. W. (2009). Plants on early alert: Glandular trichomes as sensors for insect herbivores. *New Phytologist*, *184*(3), 644–656. https://doi.org/10.1111/j.1469-8137.2009.03002.x

Quan, M., Liu, Q. Z., & Liu, Z. L. (2018). Identification of insecticidal constituents from the essential

oil from the aerial parts *Stachys riederi* var. *japonica*. *Molecules*, *23*(5), 1200. https://doi.org/10.3390/molecules23051200

Rahmani, S., & Azimi, S. (2020). Fumigant toxicity of three *Satureja* species on tomato leafminers, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Toxin Reviews*, *40*(4), 724-735. https://doi.org/10.1080/15569543.2020.1767651

Rautio, P., Markkola, A., Martel, J., Tuomi, J., Härmä, E., Kuikka, K., Siitonen, A., Riesco, I. L., & Roitto, M. (2002). Developmental plasticity in birch leaves: Defoliation causes a shift from glandular to nonglandular trichomes. *Oikos*, *98*(3), 437-446. https://doi.org/10.1034/j.1600-0706.2002.980308.x

Sá, R. D., Santana, A. S. C. O., Silva, F. C. L., Soares, L. A. L., & Randau, K. P. (2016). Anatomical and histochemical analysis of *Dysphania ambrosioides* supported by light and electron microscopy. *Revista Brasileira de Farmacognosia*, *26*(5), 533–543. https://doi.org/10.1016/j.bjp.2016.05.010

Singh, P., Jayaramaiah, R. H., Sarate, P., Thulasiram, H. V., Kulkarni, M. J., & Giri, A. P. (2014). Insecticidal potential of defense metabolites from *Ocimum kilimandscharicum* against *Helicoverpa armigera*. *PLOS One*, *9*(8), e0104377. https://doi.org/10.1371/journal.pone.0104377

Sletvold, N., Huttunen, P., Handley, R., Kärkkäinen, K., & Ågren, J. (2010). Cost of trichome production and resistance to a specialist insect herbivore in *Arabidopsis lyrata*. *Evolutionary Ecology*, *24*, 1307–1319. https://doi.org/10.1007/s10682-010-9381-6

Sneha, K., Narayanankutty, A., Job, J. T., Olatunji, O. J., Alfarhan, A., Famurewa, A. C., & Ramesh, V. (2022). Antimicrobial and larvicidal activities of different *Ocimum* essential oils extracted by ultrasound-assisted hydrodistillation. *Molecules*, *27*(5), 1456. https://doi.org/10.3390/molecules27051456

Srivastava, S., Adholeya, A., Conlan, X. A., & Cahill, D. M. (2016). Acidic potassium permanganate chemiluminescence for the determination of antioxidant potential in three cultivars of *Ocimum basilicum*. *Plant Foods for Human Nutrition*, *71*, 72-80. https://doi.org/10.1007/s11130-016-0527-8

Tian, D., Tooker, J., Peiffer, M., Chung, S. H., & Felton, G. W. (2012). Role of trichomes in defense against herbivores: comparison of herbivore response to woolly and hairless trichome mutants in tomato (*Solanum lycopersicum*). *Planta*, *236*, 1053–1066. https://doi.org/10.1007/s00425-012-1651-9

Traw, M. B., & Bergelson, J. (2003). Interactive effects of jasmonic acid, salicylic acid, and gibberellin on induction of trichomes in *Arabidopsis*. *Plant Physiology*, *133*(3), 1367–1375. https://doi.org/10.1104/pp.103.027086

van Schie, C. C. N., Haring, M. A., & Schuurink, R. C. (2007). Tomato linalool synthase is induced in trichomes by jasmonic acid. *Plant Molecular Biology*, *64*, 251–263. https://doi.org/10.1007/s11103-007-9149-8

Wang, X., Shen, C., Meng, P., Tan, G., & Lv, L. (2021). Analysis and review of trichomes in plants. *BMC Plant Biology*, *21*, 70. https://doi.org/10.1186/s12870-021-02840-x

War, A. R., Taggar, G. K., Hussain, B., Taggar, M. S., Nair, R. M., & Sharma, H. C. (2018). Special issue: using non-model systems to explore plant-pollinator and plant-herbivore interactions: plant defence against herbivory and insect adaptations. *AoB PLANTS*, *10*(4), ply037. https://doi.org/10.1093/aobpla/ply037

Xie, Z., Kapteyn, J., & Gang, D. R. (2008). A systems biology investigation of the MEP/terpenoid and shikimate/phenylpropanoid pathways points to multiple levels of metabolic control in sweet basil glandular trichomes. *The Plant Journal*, *54*(3), 349–361. https://doi.org/10.1111/j.1365-313X.2008.03429.x

Zhandi, W., La, Z., Bailian, D., Yundong, S., Yani, W., Guangqiu, L., & Lin, J. (2021). Regulation of pakchoi's secondary metabolites on the behavior of female *Plutella xylostella* (Lepidoptera: Plutellidae). *Chinese Journal of Pesticide Science*, *23*(2), 323-330. https://doi.org/10.16801/j.issn.1008-7303.2021.0016

Zhang, X. G., Li, X., Gao, Y. L., Liu, Y., Dong, W. X., & Xiao, C. (2019). Oviposition deterrents in larval frass of potato tuberworm moth, *Phthorimaea operculella* (Lepidoptera: Gelechiidae). *Neotropical Entomology*, *48*, 496-502. https://doi.org/10.1007/s13744-018-0655-y

Pertanika J. Trop. Agric. Sci. 47 (4): 1139 - 1156 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

*Review Article*

# Noni Fruit (*Morinda citrifolia* L.) Extraction and Phytochemical Analyses: A Mini Review

**Ratih Hardiyanti[1,3]*, Rochmadi[1], Muslikhin Hidayat[1] and Mohammad Affan Fajar Falah[2]**

[1]*Department of Chemical Engineering, Faculty of Engineering, Universitas Gadjah Mada, Jl. Grafika No. 2, Sleman, 55281 Yogyakarta, Indonesia*

[2]*Department of Agroindustrial Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Jl. Flora Bulaksumur No.1, Sleman, 55281 Yogyakarta, Indonesia*

[3]*Department of Bioresources Technology and Veterinary, Vocational College, Universitas Gadjah Mada, Jln. Yacaranda, Sleman, 55281 Yogyakarta, Indonesia*

## ABSTRACT

Noni fruit (*Morinda citrifolia* L.) contains many beneficial bioactive ingredients and is used in traditional medicines and health supplements in tropical and subtropical countries. However, the fruit rots easily, so it must be rapidly processed to isolate bioactive ingredients with antioxidant, antimicrobial, anti-inflammatory, and anti-cancer effects. While many different noni fruit extraction methods are available in the literature, the objective of this mini-review was to briefly assess these methods and ensure appropriate method selection for the isolation of optimal bioactive ingredients.

*Keywords*: Antioxidants, bioactive, extractions methods, *Morinda citrifolia* L., noni fruit

ARTICLE INFO

*Article history:*
Received: 18 January 2024
Accepted: 08 March 2024
Published: 27 September 2024

DOI: https://doi.org/10.47836/pjtas.47.4.06

*E-mail addresses*:
ratih.hardi@ugm.ac.id (Ratih Hardiyanti)
rochmadi@ugm.ac.id (Rochmadi)
mhidayat@ugm.ac.id (Muslikhin Hidayat)
affan_tip@ugm.ac.id (Mohammad Affan Fajar Falah)
* Corresponding author

## INTRODUCTION

De Almeida Lopes et al. (2018) used the Hawaiian term "noni" to describe the fruit of the plant species *Morinda citrifolia* Linn (from the Rubiaceae family), which is native to Australia and Southeast Asia. Noni habitats exist in tropical and subtropical climate zones such as Polynesia, India, the Caribbean, Central America, and Northern South America. Noni is taxonomically classified as Eukaryota (domain), Plantae

ISSN: 1511-3701
e-ISSN: 2231-8542

(kingdom), Spermatophyta (phylum), Angiospermae (subphylum), Dicotyledonae (class), Gentianales (order), Rubiaceae (family), and *M. citrifolia* (species) (Rojas-Sandoval, 2017). *Morinda citrifolia* has many regional names: "Indian mulberry", "nuna", or "ach" in India, "noni" in Malaysia, "nhau" in Southeast Asia, "painkiller bush" in the Caribbean, and "cheese fruit" in Australia. There are one cultivar, *M. citrifolia* cultivar *Potteri*, and two well-known variants of *M. citrifolia*: *M. citrifolia* var. *citrifolia* and *M. citrifolia* var. *bracteate* (Figure 1) (University of Hawaii at Manoa, 2006). *Morinda citrifolia* var. *citrifolia* is the most widely used variety and offers commercial and health benefits.

*Morinda citrifolia* has broad, elliptical leaves that are 5–17 cm long and 10–40 cm wide. It grows to a height of 3–10 m. The tiny, tubular white flowers are grouped on flower stalks. The petiole leaves a ring-like mark on the stem. Noni fruits are oval, fleshy, and have an embossed-like look. They are formed as syncarps by united carpels, measuring 3–10 cm long and 3–6 cm wide. Fruit ripens from green to yellow, appearing somewhat wrinkled and translucent; they nearly become white when fully ripe. Fruits weigh approximately 100–300 g, and each axillary gemma typically produces only one fruit. When ripe, the pulp has a jelly-like texture, is pale yellow or colorless, and is watery and bitter. Ripe fruits have a strong butyric acid-like odor. Small reddish-brown spheres cover the skin, containing up to 200 seeds/fruit and 3–10 mm long. The seeds of noni are slimy with a dense seed coat and contain a lot of lignin, proteins (9–15%), dissolved sugars (5%), and lipids (43–50%). Linoleic acid is the most abundant lipid fraction (10–68%) in noni seeds, and concentrations change during germination, reducing up to 38% of the total content.

Cárdenas-Coronel et al. (2016) investigated changes in harvested noni fruit quality and composition at five different phases of development, ranging from dark green to transparent gray (Table 1). Acidity, accumulated dissolved solids, and reduced pH accompanied the ripening of the fruit. Fruit-softening profiles were divided into three: early (no significant softening),

(a) (b) (c)

*Figure 1*. *Morinda citrifolia* plants: (a) *Morinda citrifolia* var. *citrifolia*, (b) *Morinda citrifolia* var. *bracteata*, and (c) *Morinda citrifolia* cv. "Potteri" (University of Hawaii at Manoa, 2006)

Table 1
*Skin color at different noni fruit ripening stages (Cárdenas-Coronel et al., 2016)*

| Ripeness stage | Subjective color and firmness (hardness in Newtons) | Fruit appearance | L* | C* | h° |
|---|---|---|---|---|---|
| 1 | Dark green, very hard (235±15 N) |  | 62.2±2.2$^{bc}$ | 38.9±1.1$^{ab}$ | 102.5±0.8$^{a}$ |
| 2 | Partially green-yellowfish, very hard (226±15 N) |  | 65.2±1.7$^{b}$ | 42.3±1.1$^{a}$ | 99.8±1.2$^{a}$ |
| 3 | Pale yellow, very hard (202±14 N) |  | 80.4±2.1$^{a}$ | 35.1±2.6$^{b}$ | 95.5±1.3$^{b}$ |
| 4 | Light yellow, moderately hard (165±12 N) |  | 81.1±1.7$^{a}$ | 30.7±1.7$^{c}$ | 89.2±2.2$^{c}$ |
| 5 | Translucent grayish, very soft (6±2N) |  | 58.5±4.3$^{c}$ | 18.0±3.7$^{d}$ | 92.7±2.4$^{b}$ |

*Note*. Mean values ± standard errors; The significant changes are denoted by different superscripted lowercase letters in the L*, C*, and h° columns (n = 3, $p$<0.05); L* = Level of light or dark; C* = Chroma; h° = Hue angle

intermediate (significant softening), and final (dramatic softening). In the early stages, extensive depolymerization of water-soluble pectin and a significant increase in pectinase activity did not correspond to a slight decrease in firmness. Intermediate stages were associated with increased pectinase and hemicellulose activity. The final stages saw the most significant reductions in alcohol-insoluble solid yields, uronic acid, and neutral sugar composition. Also, pectinase activity increased, and hemicellulose fractions were depolymerized. Thus, noni-fruit ripening occurred in line with pectinase and hemicellulose activity, which promoted the differential disassembly of cell wall polymers. Changes in soluble fruit solids are shown in Table 2.

The noni fruit juice processing industry is very dependent on the quality of the noni fruit and its juice, which means it depends on fruit production on the land. Research by Prakash et al. (2022) revealed the effects of chemical treatment on the fruiting capacity of noni, fruit yield, and antioxidant properties of the fruit. Noni plants bearing fruit are sprayed with chemicals on their leaves, namely boric acid (BA), gibberellic acid ($GA_3$), sucrose solution, and water as a control. Fruiting, fruit yield, and fruit growth rate increased significantly in plants treated with BA, $GA_3$, and sucrose compared with the control treatment. The treatment did not affect the antioxidant capacity, total soluble solids (TSS) of fruit, and total phenol content (TPC). Treatment of 20% sucrose and BA (100 and 200 ppm) showed the highest fruit yield. Table 3 shows the impact of chemical treatment on noni fruit.

Extraction method reviews can inform researchers of the best noni fruit extraction methods, e.g., reviewing method weaknesses and strengths for optimal bioactive ingredient isolation.

## NONI FRUIT

### Phytochemical Content

Noni fruit physicochemical properties were characterized by Carrillo-López and Yahia (2011) consisting of 90% water content, 3.72 of acidity, 9.87% of dry material, 8°Brix of total dissolved solids, 2.5% protein, 0.15% lipid, 8.27 g/L of fructose, 11.97 g/L of glucose, 155 mg/100 g of vitamin C, 3,900

Table 2
*Alcohol-insoluble solid (AIS) yield and uronic acid (UA) content changes in ais, cellulose compared with firmness (N), and non-cellulosic neutral sugars (NS) during noni fruit ripening (Cárdenas-Coronel et al., 2016)*

| Ripeness (stage) | Firmness (N) | AIS yield (g/100 g FW) | UA (g/100 g FW) | NS (g/100 g FW) | Cellulose (g/100 g FW) |
|---|---|---|---|---|---|
| 1 | 235.75 ± 15.61 $^{a}$ | 5.38 ± 0.80 $^{a}$ | 1.52 ± 0.06 $^{a}$ | 1.60 ± 0.17 $^{a}$ | 0.65 ± 0.07 $^{a}$ |
| 2 | 226.58 ± 15.47 $^{a}$ | 4.78 ± 0.99 $^{a}$ | 1.49 ± 0.09 $^{ab}$ | 1.50 ± 0.15 $^{a}$ | 0.65 ± 0.04 $^{a}$ |
| 3 | 202.66 ± 14.19 $^{a}$ | 4.88 ± 0.69 $^{a}$ | 1.36 ± 0.07 $^{ab}$ | 1.46 ± 0.15 $^{a}$ | 0.50 ± 0.05 $^{a}$ |
| 4 | 165.08 ± 12.50 $^{b}$ | 4.23 ± 0.36 $^{a}$ | 1.23 ± 0.05 $^{b}$ | 1.39 ± 0.21 $^{a}$ | 0.50 ± 0.10 $^{a}$ |
| 5 | 6.40 ± 3.12 $^{c}$ | 2.33 ± 0.68 $^{b}$ | 0.42 ± 0.03 $^{c}$ | 0.85 ± 0.14 $^{b}$ | 0.49 ± 0.07 $^{a}$ |

*Note*. Mean values ± standard errors; The significant differences for different letters (n = 3; $p$<0.05) in the same columns

Table 3
*Number of fruit, growth attributes, fruit yield, antioxidant capacity (AEAC), and total phenol content (TPC) in noni plants treated with different chemicals (Prakash et al., 2022)*

| Chemical treatment | Fruit Numbers per plant | Fruit growth rate (mm/day) | Mature fruit weight (g) | Fruit total soluble solids (TSS) (°Brix) | AEAC (mg/100 g FW) | TPC (mg/100 g FW) |
|---|---|---|---|---|---|---|
| Control (Water) | 5.30±0.30$^{d}$ | 0.33±0.04$^{c}$ | 215.30±8.70$^{b}$ | 7.43±0.14$^{a}$ | 506.80±14.10$^{b}$ | 150.20±1.90$^{d}$ |
| Sucrose 20% | 12.60±0.60$^{bc}$ | 0.46±0.05$^{b}$ | 287.50±6.70$^{a}$ | 7.70±0.13$^{a}$ | 313.50±11.20$^{b}$ | 125.80±1.60$^{abc}$ |
| Sucrose 5% | 10.60±0.50$^{ab}$ | 0.44±0.05$^{ab}$ | 281.40±13.90$^{a}$ | 7.62±0.11$^{a}$ | 359.70±13.80$^{a}$ | 133.40±3.50$^{bc}$ |
| BA 100 ppm | 11.00±0.80$^{ac}$ | 0.41±0.04$^{ab}$ | 265.10±13.50$^{a}$ | 7.80±0.09$^{a}$ | 330.00±10.90$^{a}$ | 119.20±2.00$^{a}$ |
| BA 200 ppm | 11.10±0.90$^{ac}$ | 0.43±0.04$^{ab}$ | 260.90±7.20$^{a}$ | 7.70±0.11$^{a}$ | 319.80±8.80$^{a}$ | 122.10±2.80$^{ab}$ |
| $GA_3$ 20 ppm | 8.60±0.40$^{a}$ | 0.40±0.04$^{a}$ | 257.70±8.70$^{ab}$ | 7.43±0.12$^{a}$ | 347.20±11.30$^{a}$ | 136.40±3.10$^{c}$ |
| $GA_3$ 40 ppm | 9.90±0.50$^{a}$ | 0.42±0.05$^{ab}$ | 271.40±13.20$^{a}$ | 7.40±0.12$^{a}$ | 346.90±11.30$^{a}$ | 132.00±3.50$^{bc}$ |

*Note.* Mean values ± standard errors
There were significant differences in:
the number of fruit: Analysis of variance (ANOVA) with F, df (6, 49) = 15.08, $p<0.0001$
the fruit growth rate: ANOVA with F, df (6, 63) = 9.951, $p\leq0.0001$
the fruit weight: ANOVA with F, df (6,91) = 4.891, $p = 0.0002$
TSS on fruit was not significantly different: ANOVA with F, df (6, 77) = 2.012, $p = 0.0740$
There is a significant difference in:
TPC: ANOVA with F, df (6, 63) = 13.83, $p<0.0001$
AEAC: ANOVA with F, df (6, 63) = 31.86, ($p<0.0001$
Different letters indicate significant differences based on Tukey's test ($p<0.05$)
BA = Boric acid; $GA_3$ = Gibberellic acid

mg/L of potassium, 214 mg/L of sodium, 14 mg/L of magnesium, and 28 mg/L calcium. The most important dry matter components were dissolved solids, dietary fiber, and protein. Aspartic acid, glutamic acid, and isoleucine were the major amino acids. 8.4% of the dry matter was minerals, particularly potassium, sulfur, calcium, and phosphorus. Ascorbic acid, 24–158 mg/100 g of dry matter, and provitamin A are the vitamins in noni. From fruit and other noni plant parts, more than 150 phytochemicals have been identified and primarily included phenolic compounds, organic acids, and alkaloids. Phenolic compounds included anthraquinones (damnacanthal, morindone, and morindin), aucubin, asperuloside, and scopoletin. The primary organic acids were caproic acid and caprylic acid. The phytochemical composition of ripe *M citrifolia* L. fruit (no seeds) is shown in Table 4.

Fifty-one phenolic compounds were identified using techniques from gas chromatography and mass spectrometry. The ripe fruit contained many carboxylic acids, especially octanoic acid (about 70% of the extracts) and hexanoic acid (about 8% of the total amount of extracts), alcohol (3-methyl-3-buten-1-ol), esters (methyl octanoate and methyl decanoate), ketones (2-heptanone), and lactones ((E)-6-dodeceno-Y-lactone). Decanoic acid, octanoic acid, and 2 E-nonenal concentrations decreased during fruit ripening, while the concentrations of several esters (methyl hexanoate, ethyl octanoate, methyl octanoate, and methyl 4 E-decenoate) increased. 3-methyl-3-buten-1-il hexanoate, unsaturated esters, and 3-methyl-3-buten-1-il octanoate were also significantly decreased during ripening (Carrillo-López & Yahia, 2011). Singh and Singh (2013) examined bioactive components in *M. citrifolia* L. fruit, leaves, and seeds, and the presence of polyphenols, tannins, flavonoids, carotenoids, ascorbic acid, nitrate, oxalic acid, and phytate has been shown. However, when water extraction was used, free radical scavenging activity (RSA) significantly inhibited 1,1-diphenyl-2-picrylhydrazyl (DPPH) ($p < 0.05$) when compared with ethanol and acetone extraction methods. The highest Ly-Tyr and Leu-enkephalin levels were found in fruit. Strong correlations ($R^2 = 0.774$; $p = 0.021$) existed between antioxidant and carotenoid activities. The bioavailability of *M. citrifolia* and its free radical scavenging activity supported its numerous health benefits. Phytochemical *M. citrifolia* fruit components and antioxidant activity are shown in Tables 5 and 6, respectively.

Table 4
*The phytochemical of* Morinda citrifolia *L. ripe fruit (with no seeds)* (*de Almeida Lopes et al., 2018)*

| Physicochemical parameters | Content |
|---|---|
| Water content (%) | 90.00–91.00 |
| Total carbohydrates (%) | 5.27–9.60 |
| Dissolved solids (°Brix) | 9.00–9.20 |
| Protein (%) | 2.36–2.50 |
| Fat (%) | 0.04–0.30 |
| Total fiber (%) | 1.00 |
| Ash content (%) | 0.66–1.34 |
| Sodium (mg/100 g) | 19.76 |
| Potassium (mg/100 g) | 3900–5012 |
| Titratable acidity (g/100 g) | 3.20–6.82 |
| pH | 3.54–4.00 |

Table 5
*Phytochemical and anti-nutrient components in* Morinda citrofolia *fruit (Singh & Singh, 2013)*

| Fruit | Phytochemical (mg/100 g) | | | | | Anti-nutrient (mg/100 g) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Phenol | Flavonoid | Tannin | Carotenoid (µg/100 g) | Vitamin C | Nitrate | Phytate | Oxalate | Saponin |
| Raw | 213.4 ± 3.7 | 266.6 ± 4.0 | 524.8 ± 3.3 | 600.0 ± 8.3 | 139.2 ± 6.0 | 17.3 ± 2.1 | 369.6 ± 5 | 29.2 ± 1.5 | 236.0 ± 3.2 |
| Ripe | 250.7 ± 7.2 | 190.0 ± 2.7 | 525.3 ± 2.6 | 515.7 ± 5.2 | 149.5 ± 6.9 | 22.0 ± 4.6 | 471.8 ± 4.8 | 37.3 ± 2.1 | 234.8 ± 1.5 |

Table 6
Morinda citrifolia *fruit extracts antioxidant activity and half maximal inhibitory concentration ($IC_{50}$) values (Singh & Singh, 2013)*

| Fruit | Antioxidant activity (%) | | | $IC_{50}$ value (µg/ml) | | |
|---|---|---|---|---|---|---|
| | Methanol extraction | Acetone extraction | Aqueous extraction | Methanol extraction | Acetone extraction | Aqueous extraction |
| Raw | 58.6 ± 0.9 | 63.9 ± 0.7 | 75.1 ± 0.6 | 75.5 ± 8.3 | 122.1 ± 5.8 | 219.9 ± 5.3 |
| Ripe | 74.4 ± 7.3 | 51.2 ± 0.2 | 72.8 ± 0.5 | 213.9 ± 64.3 | 10.2 ± 1.9 | 199.7 ± 4.4 |

Lohani et al. (2019) reported that noni fruit contained phytochemical and polysaccharide components with different biological activities. The main chemical classes were betalain, indole, glucosinolates, organosulfides, sulfides, mono or polyphenolic compounds, terpenes (isoprenoids and terpenoids), and organic acids. Bioactive functions of noni fruit components include anti-inflammatory bioactive components (such as scopoletin, quercetin, and ursolic acid), anti-dyslipidemia and anti-diabetes mellitus bioactive components (for example, deacetylasperulosic acid, oleuropein, saponins, and rutin), neuroprotective bioactive components (rutin and scopoletin), and anti-cancer bioactive components (fatty acid glycosides, iridoids, anthroquinones, and polysaccharides).

The ingredients of noni fruit include vitamins (provitamin A, ascorbic acid), phenols (scopoletin, damnacanthal), organic acids (caprylic acid, caproic acid), minerals, and amino acids (aspartic acid). *In vitro* studies using animals have shown that noni exhibits cardiovascular effects, including anti-cancer, antibacterial, anti-inflammatory, antioxidant, and analgesic properties (Chan-Blanco et al., 2006).

In accordance with the Direktorat Produksi dan Distribusi Kefarmasian (2017), noni fruit contains a minimum of 0.02% scopoletin, while thick noni fruit extracts contain a minimum of 0.38% scopoletin and no less than 10.1% (when the solvent is ethanol). Noni fruit simplicia has a minimum water-soluble extract content of 21.3% and a minimum ethanol-soluble extract of 9.8%. Riyanto and Rohman (2007) successfully isolated scopoletin from the chloroform fraction of a noni fruit methanol extract. Scopoletin exhibited a

DPPH RSA of a half maximal inhibitory concentration ($IC_{50}$) value of 348.79 µg/ml. The main constituent of *M. citrifolia* L., which contributes to its antioxidant, hepatoprotective, anti-inflammatory, and immunomodulatory properties, was proposed as scopoletin. Scopotoletin has been proposed as a biochemical marker to identify and control noni fruit quality and its derivatives (Tasfiyati et al., 2022). The phytochemical characteristics of noni juice are shown (Dussossoy et al., 2011) (Table 7).

Yang et al. (2007) reported that fresh noni fruit juice free-RSA for DPPH was 140 mg ascorbic acid equivalent (AAE)/100 ml, and total phenol was 210 mg gallic acid equivalent (GAE)/100 ml. Over 90% of free RSA was lost due to fruit fermentation for 3 months, while 20% was lost by fruit dehydration at 50°C. A free-RSA reduction of >90% was achieved when fresh noni juice was stored for 3 months at 24°C. Free-RSA decreased 10 to 55% during noni powder or noni juice storage at 4 and -18°C, respectively, for 3 months. Free-RSA reductions were much greater when noni juice or puree was treated with heat or dehydration than with total phenol reduction. Thus, noni powder or frozen fresh noni juice processing is preferable to juice fermentation to maintain substantial antioxidant properties/levels in noni fruit products.

Table 7
*Phytochemical characteristics of noni juice (Dussossoy et al., 2011)*

| Characteristics | Content |
|---|---|
| pH | 3.40 ± 0.10 |
| Dry weight[a] | 7.37 ± 0.06 |
| Dissolved solids[b] | 5.80 ± 0.00 |
| Titratable acidity[c] | 1.76 ± 0.01 |
| Fructose[d] | 2.44 ± 0.02 |
| Glucose[d] | 2.07 ± 0.01 |
| Total polyphenols[e] | 47.60 ± 2.00 |
| Total vitamin C[f] | 97.10 ± 2.30 |
| Dehydroascorbate acid[f] | 26.00 ± 0.80 |
| Ascorbate acid[f] | 71.10 ± 1.40 |

*Note*. Mean values ± standard errors of three experiments; [a] = %/fresh weight; [b] = Degree Brix; [c] = g citric acid/100 g fresh weight; [d] = g/100 g fresh weight; [e] = mg gallic acid equivalent/100 g fresh weight; [f] = mg/100 g fresh weight

Guo et al. (2020) investigated noni fruit and fermented juice antioxidant activity in an *in vitro* study. When compared with fermented juice, fresh fruit had a higher antioxidant capacity. However, fermented juice exerted more preventive effects on alcohol-induced acute liver injury, based on liver function and structural integrity measurements. Thus, noni juice exhibited hepatoprotective effects.

Sina et al. (2021) compared the phytochemical content of noni fruit juice obtained by squeezing noni fruit and fruit juice that had been fermented for two months. Both treatments showed the presence of flavonoids, alkaloids, catechic tannin, saponosids, gallic tannin, reducing compounds, *o*-heteroside, and leuco-anthocyanin. The antiradical activity of fresh fruit juice (41.67%) was higher than that of fermented fruit juice (21.28%), even compared to ascorbic acid (37%). The best concentration required to inhibit 50% of DPPH radicals is fresh fruit (0.024 mg/ml), followed by ascorbic acid (0.027 mg/ml) and fermented fruit (0.047 mg/ml).

Another study reported that noni fruit extract powder, generated by spray drying, showed 28.36% DPPH inhibitory activity. Thus, fruit extract powder could be used as a food additive or raw material in foods (Krishnaiah et al., 2012). Yang et al. (2010) investigated the antioxidant levels, ascorbic acid, and total phenolic content of noni juice and noni powder stored at 24°C. After two weeks of storage, noni juice exposed to light had lost 32% of total phenolic content, 89% of ascorbic acid, and 46–65% of antioxidant capacity, or approximately 8, 22, and 9–15% more when compared with juice that not exposed to light. After four weeks, 97% of ascorbic acid had been lost in the light-exposed and non-light-exposed juices. The properties of antioxidants in light and non-exposed juices had not changed significantly at 12 weeks. After 12 weeks, noni powder exposed to light had lost 21% total phenol, 17% ascorbic acid, and 23–36% antioxidant capacity or approximately 13, 4, and 7–19% more when compared with powder not exposed to light. Thus, superior powder antioxidant properties in brown packaging were maintained over and above the properties seen in transparent packaging. The degradation of antioxidant properties of noni juice has been reduced effectively for 2 weeks because of protection from light, whereas it took 3 months for noni powder.

Ruhomally et al. (2016) reported that in ripe noni fruit, there was 76.24 ± 1.13 mg/100 g ascorbic acid content, 748.40 ± 8.85 µg GAE/g fresh weight (FW) of TPC in ripe fruit extracts, while 770.34 ± 2.27 µg GAE/g FW of TPC in raw fruit extract. Both ripe and unripe noni fruit extracts strongly inhibit nitric oxide, hydroxyl radicals, and superoxide. Iron reduction capacity ranged from 11.26 ± 0.33 to 11.90 ± 0.20 mM $Fe^{2+}$/g FW, while the $IC_{50}$ values for iron (II) chelating activity of ripe and unripe fruit extract were 0.50 ± 0.01 and 1.74 ± 0.01 g FW/ml, respectively.

Noni fruit contains many bioactive substances, including polyphenols and flavonoids, which provide antioxidant benefits for human health. Thus, optimal bioactive ingredient extraction is required to collect and purify these ingredients.

## Fruit Extraction

Solid-liquid extraction is a heterogeneous process that involves transferring solutes from a solid to a liquid. Complex solute combinations, such as extractable plant chemicals, can be extracted at different degrees depending on plant location (outer surface, pores, or vacuoles). The following five steps are involved in solvent transfer processes: (1) solvent from the liquid solvent to the surface of the extracted particles, (2) solvent diffusion into the solid matrix, (3) component dissolution, (4) solvent transfer through the solid matrix, and (5) solvent transfer from the solid's outer surface to the liquid solvent (Conde et al., 2010).

According to Seader et al. (2011), leaching, also known as solid-liquid (or liquid-solid) extraction, requires a liquid solvent to separate soluble components (solute or leachate) from a solid material.

The solute diffuses from the solid into the solvent around it. The desired result can be either an extracted solid fraction, an insoluble solid, or both. Leaching can be conducted using batch, semi-continuous, or continuous processes. Leaching stage outputs comprise a liquid-free solid material called "overflow" and a wet solid called "underflow". Leaching is followed by washing to reduce solute content in the liquid phase of the underflow. These processes generate a final underflow, the extracted solid moistened with an almost pure solvent, and a final overflow, or an extract containing part of the solvent and most of the solute.

Because solute concentrations in solids vary during extraction processes, the rates at which extracts are obtained are not linear with respect to time. It causes unstable or non-stationary conditions. During interaction times between particles containing solids and the extraction solvent, several phenomenological events occur (Veggi et al., 2013):

1. Solvent penetration into the solid matrix
2. Component dissolution and/or breakdown
3. Solute transport out of the solid matrix and extracted solute migration from the outer solid surface into the solution.

### *Near Supercritical Fluid Carbon Dioxide Extraction (SF-$CO_2$)*

Chen et al. (2009) extracted noni fruit using the near SF-$CO_2$ method. Fresh fruit was washed in running water, cut into pieces, dried for 5 days in an oven at 37°C, ground to a powder, and sifted through a 20 mesh. The powder moisture content was <2%. The powders were stored at -80°C in plastic bottles to limit the damage. A 50 ml stainless steel vessel has been used as the extraction chamber. Near SF-$CO_2$ was performed at 35 or 50°C and 1,500 or 3,500 psi pressure for 3 hr statically, hereafter a dynamic extraction for 1 hr. The vessel was filled with 10 g of sample, and the extracted analyte was accommodated in a 20 ml measuring flask containing 5 ml of absolute ethanol. An ice bath was used to enhance dynamic extraction in a 20 ml volumetric flask. The extracted samples were re-suspended in 100% ethanol, dried at ambient temperature, and kept at -80°C. Using the constant weight method, the sample weight after extraction was divided by the initial weight (10 g) to generate extraction data. Before analyzing antioxidant activity, samples were re-suspended in absolute ethanol and centrifuged for 10 min at 4°C and 8,000 x *g*. For antioxidant activity tests, supernatants were gathered and serially diluted (the final ethanol concentration was less than 1%). Extracted contents and antioxidant activities are summarized in Table 8.

### *Ethanol Extraction*

Thoo et al. (2010) extracted dried *M. citrifolia* fruit powder to generate phenolic antioxidants using 0 to 100% (v/v) of ethanol solvents, 20 to 120 min of extraction times, and 25 to 65°C of extraction temperatures. Dried fruit powder (2 g) was extracted in

Table 8
*Phenol content and antioxidant activity of noni fruit extracts (Chen et al., 2009)*

| Extraction conditions | Total phenolic content (mg/g) | Antioxidative activity ($IC_{50}$) | | | |
|---|---|---|---|---|---|
| | | DPPH radical scavenging activity (mg/L) | Hydroxyl radical scavenging activity (mg/L) | Hydrogen peroxide scavenging activity (g/L) | Ferrous ion chelating activity (g/L) |
| 3,500 psi; 35°C (method A) | 2.59 ± 0.08 | 157.07 ± 3.26 | Undefined | Undefined | Undefined |
| 3,500 psi; 50°C (method B) | 6.14 ± 0.41[a] | 373.97 ± 6.59[a] | Undefined | 2.73 ± 0.65 | Undefined |
| 1,500 psi; 35°C (method C) | 0.49 ± 0.004[a,b] | 597.26 ± 24.43[a,b] | Undefined | 1.03 ± 0.05 | Undefined |
| 1,500 psi; 50°C (method D) | 14.82 ± 0.83[a,b,c] | 651.81 ± 24.03[a,b,c] | Undefined | Undefined | Undefined |

*Note*. Mean values ± standard errors; $IC_{50}$ = Effective concentration to deliver 50% response; Undefined indicates very low values/no inhibitory activity because $IC_{50}$ values were too large to be detected; [a] = Significant difference between this method and method A; [b] = Significant difference between this method and method B; [c] = Significant difference between this method and method C

20 ml solvent in a temperature-controlled shaker or water bath at a constant speed and specific temperature. The crude extract was refined through Whatman No. 1 filter paper, after which antioxidant compound and antioxidant capacity levels were assessed. The results demonstrated that the extraction conditions significantly impacted antioxidant capacity and phenolic compounds. With a TPC value of 919.95 mg GAE/100 g DW, a TFC of 472.73 mg CE/100 g DW, a 2,20-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) inhibition of 791.71 µmol Trolox equivalent antioxidant capacity (TEAC)/100 g DW, and a DPPH inhibition of 1928.5 µmol TEAC/100 g DW, the ideal extraction conditions were 40% ethanol for 80 min at 65°C. Depending on the extraction duration ($r$ = 0.938) and ethanol content ($r$ = 0.932), TPC, and DPPH showed a substantial correlation.

### *Ultrasonic-Assisted Extraction (UAA), Pulsed Electric Field-Assisted Extraction (PEFAE), and Hot Water Extraction (HWE)*

Li et al. (2020) compared the effects of different methods of extraction on physicochemical characteristics, polysaccharide extraction yield, antiproliferative capabilities, and antioxidant activity of *M. citrifolia* L. Three extraction methods were compared: HWE, PEFAE, and UAA. Fully ripe noni fruits (brown and soft) were washed and dried at 55°C, then dried into powder and passed through a 60-mesh sieve. A Soxhlet system (75°C for 6 hr) using ethanol (90%, v/v) has been used to remove grease and impurities from the powder. The residue was dehydrated at 50°C for 48 hr after filtration. UEA generated the highest extraction yields, smallest

molecular weights, best antioxidant activity, and excellent antiproliferative abilities. Antioxidant activity values represented that UAE, HWE, and PEFAE donated hydrogens to inhibit DPPH radicals. However, UAE extracts had better ABTS radical inhibitory activity when compared with HWE and PEFAE. Also, UAE extracts had better hydroxyl radical inhibitory activity when compared with HWE and PEFAE methods.

### *High Hydrostatic Pressure Extraction (HHPE)*

Jamaludin et al. (2020) extracted noni fruit using the HHPE method. The fruit was washed, sliced thinly, freeze-dried, ground, and sieved using a 20–50 mesh sieve. A high-pressure food processor (Frescal MFP-7000, Mitsubishi Heavy Ind., Japan) was used to perform HHPE; 1 g dry fruit powder and 30 ml extraction solvent (ethanol solution) were placed into a polyethylene bag, then placed into another polyethylene (PE) bag, and vacuum sealed. The HHPE was operated at room temperature, and water was poured into the high hydrostatic pressure (HHP) vessel as a pressure transfer medium. A hydraulic pump generated pressure and maintained the required processing time. After the pressure was released, the PE bag was removed, the extract was filtered, and the filtrate was diluted in methanol and analyzed for bioactive content.

The effects of pressure, ethanol concentration, and time on bioactive compound extraction were individually evaluated. The ethanol concentration was varied from 30 to 70% (v/v) at 20% intervals; the extraction pressure was changed from 400 to 600 MPa at 100 MPa intervals; and extraction times varied from 5–15 min at 5 min intervals.

Extraction parameters were maintained at a moderate value if not reviewed: 50%, 500 MPa, and 10 min. Bioactive compounds that were methanol-extracted from noni fruit were: 533.4 ± 33.1 μg/g dry sample of scopoletin, 544.9 ± 21.9 μg/g dry sample of rutin, and 23.4 ± 1.0 μg/g dry sample of alizarin. The response surface methodology and the Box–Behnken design have been used to examine optimum extraction conditions. Extraction pressure and ethanol concentrations were the most statistically significant variables that produced high extraction yields. Optimal HHPE extraction conditions (>0.998 desired level) for simultaneous rutin, alizarin, and scopoletin extraction were 65% concentration of ethanol, 544 MPa, and 15 min, which generated maximum yields of 82.4% scopoletin 77.2% alizarin, and 82.2% rutin (Jamaludin et al., 2020).

### *High-pressure Extractor (HPE) Method*

Krishnaiah et al. (2015) extracted noni fruit using the HPE method. Fresh seedless fruit were washed, cut into pieces, dried in an oven at 60°C for 2 days, then ground to powder. The powder was extracted in methanol using an HPE at specific temperature, pressure, and time values. The supernatant was separated from the powder residue by filtering through Whatman filter paper No. 4 and evaporated (at reduced pressure) to

generate a dark green viscous material. TPC = 43.18 mg GAE/10 g and DPPH radical inhibitory activity = 55.60% values were generated at 60°C, 1.5 bar pressure, and 6 hr extraction conditions.

### *Accelerated Solvent Extraction (ASE)*

Tasfiyati et al. (2022) used ASE on noni fruit to generate scopoletin. Ripe fruit was peeled, cut into pieces, dried in an oven at 50°C for several days, ground to a powder, and filtered through an 80-mesh sieve. The powder was extracted using ASE on an automated Energized Dispersive Guided Extraction® system (CEM Corporation, USA): 1 g powder was placed in a stainless-steel sample holder with cellulose filter paper 2.5 µm. The solvent (ethanol) flowed into the sample chamber at a specified time and temperature. The extract flowed into a container through a cooling coil (at the bottom of the sample chamber). Optimum scopoletin levels were generated for 12 min at 60°C in ethanol and 1:30 (w/v) for the ratio of solid-to-solvent. After 12 min of ASE, scopoletin levels were 377.30 ± 5.27 µg/g, whereas, after a 24 hr maceration extraction process, 244.56 ± 37.31 µg/g was generated.

### *Solvent Variation*

Chang-hong et al. (2007) extracted freeze-dried fermented noni juice using three solvents: ethyl acetate (EtOAc), 1-butanol (n-BuOH), and petroleum ether. Three phenolic antioxidant components were isolated from the EtOAc solvent: aesculetin, 3,3',4',5,7-pentahydroxyflavone (quercetin), and isoscopoletin. The EtOAc solvent extraction showed higher antioxidant activity when compared with mannitol/ vitamin C, whereas petroleum ether and n-BuOH solvent extractions had less activity besides mannitol.

### *High-Performance Liquid Chromatography (HPLC) Extraction*

A quantitative scopoletin analytical method using noni fruit water extraction (aqueous fruit extracts [AFE]) was formulated by Mahattanadul et al. (2011). Diluted fruit extracts were analyzed using the high-performance liquid chromatography with ultra-violet (HPLC-UV) spectroscopy method. A mobile phase (isocratic mixture of 0.01 M sodium acetate: acetonitrile [80:20, v/v]) was operated at 1.0 ml/min, and scopoletin content in eluents was monitored at 350 nm. A calibration curve of several scopoletin concentrations (0.05–10 µg/ml) was used to calculate AFE scopoletin concentrations. Comparing retention times (5.585 min) with a scopoletin standard (5.497 min) performed identification of scopoletin peak on AFE chromatograms. The scopoletin content was 0.85–0.87 mg in 1 g of lyophilized AFE powder.

De Moraes et al. (2019) performed HPLC to determine flavonoid and phenolic ingredients in *M. citrifolia* AFE. From chromatograms, *M. citrifolia* AFE contained (2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxypropanoic acid (6), tR 37.5 min; 2,3,7,8-Tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione (5), tR 35 min; 2-(3,4-dihydroxyphenyl)-

5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl] oxan-2 yl]oxychromen-4-one (4), tR 32.5 min; (E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid (3), tR 15.1 min; (1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid (2), tR 14.9 min; and phenolic compounds (e.g., gallic acid, chlorogenic acid, caffeic acid, ellagic acid, and rosmarinic acid), flavonoids (rutin) (e.g. 3,4,5-trihydroxybenzoic acid (1), tR 5 min.

Phenolic acid and flavonoid levels in noni juice (made from fermented and pasteurized fruit) were also examined by HPLC (Lin et al., 2013). The UV spectra were recorded between 220 and 450 nm. Bioactive content is shown in Table 9.

The minor element of noni juice was analyzed using high-performance liquid chromatography coupled with diode-array detection and electrospray ionization tandem mass spectrometry (HPLC-DAD-$MS^n$) (Dussossoy et al., 2011). Nine elements were quantified, and twelve were identified. Several phenolic compounds were determined: phenolic acids (vanillic acid), coumarins (scopoletin and esculetin), vanillin and iridoids (asperulosidic acid and deacetylasperulosidic acid), and flavonoids (rutin, kaempferol rutinoside, quercetin, isoquercitrin, and quercetin derivatives). Asperulosidic acid (71.6 mg equivalent of loganic acid/100 g FW) and deacetylasperulosidic acid (159.1 mg equivalent of loganic acid/100 g FW) were the major elements in noni juice. The main polyphenol compounds were scopoletin 1.33 mg/100 g FW and rutin 4.63 mg/100 g FW. Phenolic and iridoid compound quantities are shown in Table 10.

Table 9
*Flavonoid and phenolic acid content in noni fruit juice (Lin et al., 2013)*

| Component | Content (mg/100 ml juice) |
|---|---|
| Gallic acid | 1.79 ± 0.01 |
| Gentisic acid | 19.16 ± 0.75 |
| Chlorogenic acid | 10.49 ± 0.01 |
| *p*-hydroxybenzoic acid | 14.12 ± 0.42 |
| Caffeic acid | 5.42 ± 0.02 |
| Epicatechin | 2.94 ± 0.03 |
| Ferulic acid | 3.36 ± 0.01 |
| *p*-anisic acid | 5.07 ± 0.03 |
| Naringin | 3.39 ± 0.02 |
| Hesperidin | 3.85 ± 0.02 |
| Phenolic acid content | 59.41 ± 1.22 |
| Flavonoid content | 10.61 ± 0.04 |
| Total | 69.59 ± 1.26 |

Table 10
*Noni juice components (Dussossoy et al., 2011)*

| Component | Amount (mg/100 g FW) |
|---|---|
| Quercetin | 0.29 ± 0.01[a] |
| Quercetin derivative | 0.46 ± 0.02[a] |
| Scopoletin | 1.32 ± 0.02[a] |
| Rutin | 4.63 ± 0.04[a] |
| Esculetin | 0.20 ± 0.01[a] |
| Isoquercitrin | tr[b] |
| Desacetylasperulosidic acid | 159.1 ± 8.1[a] |
| Asperulosidic acid | 71.6 ± 4.1[a] |
| Vanillic acid | 0.26 ± 0.00[a] |
| Vanillin | 0.35 ± 0.01[a] |
| Kaempferol derivative | tr[b] |
| Protocatechuic acid | tr[b] |

*Note*. [a]Mean values ± standard errors of three independent assessments
[b]tr = Trace amounts (<0.1 mg/100 g fresh weight)

Wu et al. (2019) reported that the compounds of fresh noni fruit that related to antioxidant activity were D-tagatose, ethylsuberenol, serotinose, (R)-3, 7-dihydroxy-2, 4-dimethoxyisoflavan, ancistrotectorine, 3-hydroxy-5Z-octenyl acetate, garcimangosone C, (−)-epigallocatechin, eugenitin, musanolone C, archange-slippery, and *O*-isopentenylhalfordinol. The component that significantly increased after harvesting were DL-malic acid, palmitic acid, isooctyl acetate, marmelolactone A, isorhamnetin 3-(6-malonylglucoside), ethylparaben, ethyl (4Z)-4,7-octadienoate, coumarin, geranylacetone, (Z)-alpha-irone, cedrelanol, carotol, norecasantalol, 5-dodecyldihydro-2(3H)-furanone, (2E,4E)-2,4-dodecadienal, and BRXanthone A. These molecules may account for the antibacterial properties of noni fruit and their benefits toward the immune system and inflammation.

Extraction processes break down protective cells to release bioactive ingredients so they can be separated and purified. Physical (e.g., pressure, temperature, or electromagnetic waves), chemical extraction techniques (e.g., solvents that bind to bioactive ingredients), or a combination thereof have been successfully applied to noni fruit. Such extraction methods must minimize the impact on bioactive ingredient activity, e.g., polyphenols and flavonoids, which have multiple health benefits. Extraction methods should be selected based on equipment availability, extraction time, optimal extraction content, and environmental safety (e.g., waste produced).

A variety of extraction methods have been applied to noni fruit to obtain the optimum amount of bioactive substances. Various operating conditions, such as temperatures below 70°C, continue to be considered by conventional extraction methods that are used up to high pressure. It is intended to ensure that the extracted bioactive materials, for example, polyphenols and flavonoids with antioxidant functions, are not damaged. Solvent selection leads to solvents that are safe for the extracted material and the environment, for example, ethanol, which is polar and suitable for polyphenols and flavonoid extraction. With additional treatments, such as pressure, the use of solvents is gradually reduced.

The use of advanced equipment encourages the provision of funding, which is a major weakness in today's extraction process. However, this weakness may be overcome by maximizing the extraction results and making full use of them to minimize the loss of bioactive materials in noni fruit.

## CONCLUSION

Noni fruit contains phytochemical components that have multiple health benefits. Several extraction techniques are available that can generate optimally bioactive extracts. The separation technique for noni fruit was solid-liquid extraction or leaching. Polyphenols and flavonoids, which are highly beneficial for health, are bioactive from noni fruit.

## ACKNOWLEDGMENTS

This research was supported by the Indonesia Endowment Fund for Education Agency (Lembaga Pengelola Dana Pendidikan) Scholarship 2020, Ministry of Finance, Republic of Indonesia (LOG 1423202082025763).

## REFERENCES

Cárdenas-Coronel, W. G., Carrillo-López, A., de La Rocha, R. V., Labavitch, J. M., Báez-Sañudo, M. A., Heredia, J. B., Zazueta-Morales, J. J., Vega-García, M. O., & Sañudo-Barajas, J. A. (2016). Biochemistry and cell wall changes associated with noni (*Morinda citrifolia* L.) fruit ripening. *Journal of Agricultural and Food Chemistry*, *64*(1), 302–309. https://doi.org/10.1021/acs.jafc.5b03681

Carrillo-López, A., & Yahia, E. M. (2011). Noni (*Morinda citrifolia* L.). In E. M. Yahia (Ed.), *Postharvest biology and technology of tropical and subtropical fruits: Mangosteen to white sapote* (pp. 51–62). Woodhead Publishing. https://doi.org/10.1533/9780857092618.51

Chan-Blanco, Y., Vaillant, F., Perez, A. M., Reynes, M., Brillouet, J.-M., & Brat, P. (2006). The noni fruit (*Morinda citrifolia* L.): A review of agricultural research, nutritional and therapeutic properties. *Journal of Food Composition and Analysis*, *19*(6–7), 645–654. https://doi.org/10.1016/j.jfca.2005.10.001

Chang-hong, L., Ya-rong, X., Yong-hang, Y., Feng-feng, Y., Jun-yan, L., & Jing-lei, S. (2007). Extraction and characterization of antioxidant compositions from fermented fruits juice of *Morinda citrifolia* (noni). *Agricultural Sciences in China*, *6*(12), 1494–1501. https://doi.org/10.1016/S1671-2927(08)60013-9

Chen, C.-H., Lin, T.-P., Chung, Y.-L., Lee, C.-K., Yeh, D.-B., & Chen, S.-Y. (2009). Determination of antioxidative properties of *Morinda citrifolia* using near supercritical fluid extraction. *Journal of Food and Drug Analysis*, *17*(5), 7. https://doi.org/10.38212/2224-6614.2592

Conde, E., Moure, A., Domínguez, H., & Parajó, J. C. (2010). Extraction of natural antioxidants from plant foods. In S. S. H. Rizvi (Ed.), *Separation, extraction and concentration processes in the food, beverage and nutraceutical industries* (pp. 506–594). Woodhead Publishing. https://doi.org/10.1533/9780857090751.2.506

de Almeida Lopes, M. M., Sanches, A. G., de Sousa, J. A., & de Oliveira Silva, E. (2018). Noni — *Morinda citrifolia* L. In S. Rodrigues, E. de O. Silva, & E. S. de Brito (Eds.), *Exotic fruits* (pp. 319–325). Academic Press. https://doi.org/10.1016/B978-0-12-803138-4.00042-3

de Moraes, G. P., de Alencar, M. V. O. B., da Silva Araújo, L., Gomes, D. C. V., Paz, M. F. C. J., Islam, M. T., de Carvalho Melo-Cavalcante, A. A., & Picada, J. N. (2019). Cytogenotoxic study of aqueous fruit extract of *Morinda citrifolia* in Wistar albino rats. *Oriental Pharmacy and Experimental Medicine*, *19*, 311–321. https://doi.org/10.1007/s13596-019-00358-0

Direktorat Produksi dan Distribusi Kefarmasian. (2017). *Farmakope herbal Indonesia* [Pharmacopoeia of Indonesia herbs] (2nd ed.). Direktorat Produksi dan Distribusi Kefarmasian.

Dussossoy, E., Brat, P., Bony, E., Boudard, F., Poucheret, P., Mertz, C., Giaimis, J., & Michel, A. (2011). Characterization, anti-oxidative and anti-inflammatory effects of Costa Rican noni juice (*Morinda citrifolia* L.). *Journal of Ethnopharmacology*, *133*(1), 108–115. https://doi.org/10.1016/j.jep.2010.08.063

Guo, M., Mao, B., Sadiq, F. A., Hao, Y., Cui, S., Yi, M., Hong, Q., Lee, Y.-K., & Zhao, J. (2020). Effects of noni fruit and fermented noni juice against acute alcohol induced liver injury in mice. *Journal of Functional Foods*, *70*, 103995. https://doi.org/10.1016/j.jff.2020.103995

Jamaludin, R., Kim, D.-S., Md Salleh, L., & Lim, S.-B. (2020). Optimization of high hydrostatic pressure extraction of bioactive compounds from noni fruits. *Journal of Food Measurement and Characterization*, *14*, 2810–2818. https://doi.org/10.1007/s11694-020-00526-w

Krishnaiah, D., Bono, A., Sarbatly, R., & Anisuzzaman, S. M. (2015). Antioxidant activity and total phenolic content of an isolated *Morinda citrifolia* L. methanolic extract from poly-ethersulphone (PES) membrane separator. *Journal of King Saud University - Engineering Sciences*, *27*(1), 63–67. https://doi.org/10.1016/j.jksues.2013.01.002

Krishnaiah, D., Sarbatly, R., & Nithyanandam, R. (2012). Microencapsulation of *Morinda citrifolia* L. extract by spray-drying. *Chemical Engineering Research and Design*, *90*(5), 622–632. https://doi.org/10.1016/j.cherd.2011.09.003

Li, J., Niu, D., Zhang, Y., & Zeng, X.-A. (2020). Physicochemical properties, antioxidant and antiproliferative activities of polysaccharides from *Morinda citrifolia* L. (Noni) based on different extraction methods. *International Journal of Biological Macromolecules*, *150*, 114–121. https://doi.org/10.1016/j.ijbiomac.2019.12.157

Lin, Y.-L., Chang, Y.-Y., Yang, D.-J., Tzang, B.-S., & Chen, Y.-C. (2013). Beneficial effects of noni (*Morinda citrifolia* L.) juice on livers of high-fat dietary hamsters. *Food Chemistry*, *140*(1–2), 31–38. https://doi.org/10.1016/j.foodchem.2013.02.035

Lohani, M., Majrashi, M., Govindarajulu, M., Patel, M., Ramesh, S., Bhattacharya, D., Joshi, S., Fadan, M., Nadar, R., Darien, B., Maurice, D. V., Kemppainen, B., & Dhanasekaran, M. (2019). Immunomodulatory actions of a Polynesian herb Noni (*Morinda citrifolia*) and its clinical applications. *Complementary Therapies in Medicine*, *47*, 102206. https://doi.org/10.1016/j.ctim.2019.102206

Mahattanadul, S., Ridtitid, W., Nima, S., Phdoongsombut, N., Ratanasuwon, P., & Kasiwong, S. (2011). Effects of *Morinda citrifolia* aqueous fruit extract and its biomarker scopoletin on reflux esophagitis and gastric ulcer in rats. *Journal of Ethnopharmacology*, *134*(2), 243–250. https://doi.org/10.1016/j.jep.2010.12.004

Prakash, R., Jokhan, A. D., & Singh, R. (2022). Effects of foliar application of gibberellic acid, boric acid and sucrose on noni (*M. citrifolia* L.) fruit growth and quality. *Scientia Horticulturae*, *301*, 111098. https://doi.org/10.1016/j.scienta.2022.111098

Riyanto, S., & Rohman, A. (2007). Isolasi skopoletin dari buah mengkudu (*Morinda citrifolia* L.) dan uji aktivitas antioksidannya [Isolation of scopoletin from noni fruit (*Morinda citrifolia* L.) and its antioxidant activity test]. *Agritech*, *27*(3), 107–111. https://doi.org/10.22146/agritech.9598

Rojas-Sandoval, J. (2017). Morinda citrifolia *(Indian mulberry)*. CABI Compendium. https://doi.org/10.1079/cabicompendium.34854

Ruhomally, Z., Somanah, J., Bahorun, T., & Neergheen-Bhujun, V. S. (2016). *Morinda citrifolia* L. fruit extracts modulates $H_2O_2$-induced oxidative stress in human liposarcoma SW872 cells. *Journal of Traditional and Complementary Medicine*, *6*(3), 299–304. https://doi.org/10.1016/j.jtcme.2015.09.003

Seader, J. D., Henley, E. J., & Roper, D. K. (2011). *Separation process principles: Chemical and biochemical operations* (3rd ed.). Wiley.

Sina, H., Dramane, G., Tchekounou, P., Assogba, M. F., Chabi-Sika, K., Boya, B., Socohou, A., Adjanohoun, A., & Baba-Moussa, L. (2021). Phytochemical composition and *in vitro* biological activities of *Morinda citrifolia* fruit juice. *Saudi Journal of Biological Sciences*, *28*(2), 1331–1335. https://doi.org/10.1016/j.sjbs.2020.11.059

Singh, D. R., & Singh, S. (2013). Phytochemicals in plant parts of noni (*Morinda citrifolia* L.) with special reference to fatty acid profiles of seeds. *Proceedings of the National Academy of Sciences, Inia Section B: Biological Sciences*, *83*, 471–478. https://doi.org/10.1007/s40011-013-0154-1

Tasfiyati, A. N., Antika, L. D., Dewi, R. T., Septama, A. W., Sabarudin, A., & Ernawati, T. (2022). An experimental design approach for the optimization of scopoletin extraction from *Morinda citrifolia* L. using accelerated solvent extraction. *Talanta*, *238*(Part 1), 123010. https://doi.org/10.1016/j.talanta.2021.123010

Thoo, Y. Y., Ho, S. K., Liang, J. Y., Ho, C. W., & Tan, C. P. (2010). Effects of binary solvent extraction system, extraction time and extraction temperature on phenolic antioxidants and antioxidant capacity from mengkudu (*Morinda citrifolia*). *Food Chemistry*, *120*(1), 290–295. https://doi.org/10.1016/j.foodchem.2009.09.064

University of Hawaii at Manoa. (2006). *The noni website: Botany*. https://www.ctahr.hawaii.edu/noni/botany.asp

Veggi, P. C., Martinez, J., & Meireles, M. A. A. (2013). Fundamentals of microwave extraction. In F. Chemat & G. Cravotto (Eds.), *Microwave-assisted extraction for bioactive compounds: Theory and practice* (pp. 15–52). Springer. https://doi.org/10.1007/978-1-4614-4830-3_2

Wu, T., Li, M., & Lan, Z. (2019). Reveal the variation patterns of chemical composition in the fruit of *Morinda citrifolia* (noni) during postharvest storage through metabolomic characterization. *Tropical Plant Biology*, *12*, 85–97. https://doi.org/10.1007/s12042-019-09222-6

Yang, J., Gadi, R., Paulino, R., & Thomson, T. (2010). Total phenolics, ascorbic acid, and antioxidant capacity of noni (*Morinda citrifolia* L.) juice and powder as affected by illumination during storage. *Food Chemistry*, *122*(3), 627–632. https://doi.org/10.1016/j.foodchem.2010.03.022

Yang, J., Paulino, R., Janke-Stedronsky, S., & Abawi, F. (2007). Free-radical-scavenging activity and total phenols of noni (*Morinda citrifolia* L.) juice and powder in processing and storage. *Food Chemistry*, *102*(1), 302–308. https://doi.org/10.1016/j.foodchem.2006.05.020

Pertanika J. Trop. Agri. Sci. 47 (4): 1157 - 1174 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Effect of Different Drying Methods on Colour, Total Phenolic Content, Flavonoid Content, and Antioxidant Activity Retention of *Strobilanthes crispus* Leaves

**Iman Nur Sabrina Norasmadi[1], Nurain Nabilah Zulkipli[1], Suhaizan Lob[1], Wan Zawiah Wan Abdullah[1,2], Mohd Fauzi Jusoh[1] and Aidilla Mubarak[1,2]***

[1]*Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia*

[2]*Research Program of Postharvest Research and Innovation, Food Security Research Cluster, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia*

## ABSTRACT

*Strobilanthes crispus*, a medicinal herb, is recognised for its abundant phytochemicals, notably in its leaves, contributing to its high antioxidant activity. However, the crucial step of drying, aimed at extending shelf life, can impact the stability of these bioactive compounds. This study evaluates the impact of different drying methods, which include oven, microwave, freeze drying, and air drying, on the colour, phenolic and flavonoid content, and antioxidant activities of *S. crispus* leaves. The colour analysis of the fresh and dried leaves was assessed using the chromameter. Total phenolic content (TPC) and total flavonoid content (TFC) were determined using Folin-Ciocalteu's and aluminium chloride colourimetric assays, respectively. Antioxidant capacities were analysed via ferric-reducing antioxidant power (FRAP) and a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The results showed that microwave-dried *S. crispus* leaves exhibited minimal alterations in colour attributes $L^*$, $a^*$, and $b^*$, closely resembling the fresh leaves ($p > 0.05$). Microwave drying significantly preserved TPC (145.42 ± 1.61 mg GAE/g), TFC (117.27 ± 5.10 mg QE/g), FRAP activity (258.92 ± 0.15 µg TE/g extract), and displayed the most potent DPPH scavenging half-maximal inhibitory concentration (7.58 ± 0.48 µg/ml) compared to other methods ($p < 0.05$). Notably, the DPPH scavenging potency surpassed that of the synthetic

ARTICLE INFO

*Article history:*
Received: 24 January 2024
Accepted: 13 March 2024
Published: 02 October 2024

DOI: https://doi.org/10.47836/pjtas.47.4.07

E-mail addresses:
imannursabrina.2098@gmail.com (Iman Nur Sabrina Norasmadi)
nurainnabilahzulkipli@gmail.com (Nurain Nabilah Zulkipli)
suhaizanlob@umt.edu.my (Suhaizan Lob)
wzawiah@umt.edu.my (Wan Zawiah Wan Abdullah)
pozie@umt.edu.my (Fauzi Jusoh)
aidilla@umt.edu.my (Aidilla Mubarak)
*Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

antioxidant butylated hydroxytoluene. In conclusion, microwave drying appeared to be an efficient method for preserving the colour and antioxidant properties of *S. crispus* leaves. It highlights its potential as a favourable drying technique for conserving bioactive compounds in medicinal plant materials, offering promising applications in the nutraceutical and pharmaceutical fields.

*Keywords*: Bioactive compounds, leaves drying methods, medicinal plant, microwave drying, phytochemicals, *Strobilanthes crispus*

## INTRODUCTION

*Strobilanthes crispus*, locally known as *pecah kaca*, is one of the traditional folklore herbal medicines found locally in Malaysia and Indonesia. It is used as an infusion or concoction and is intended for treatments as antidiabetic, anticancer, antilytic, diuretic, and laxative agents (Nurraihana & Norfarizan-Hanoon, 2013). The leaf extract of *S. crispus* has been reported for its pharmacological properties, such as reducing the glucose level in the blood (antihyperglycemic), improving the lipid profile (antilipidemic), and lowering the threat of cardiovascular diseases (Mohd Fadzelly et al., 2006). This plant has further displayed notable anticancer activity when it was reported to inhibit cancer cell proliferation (Bakar et al., 2006) and antibacterial properties as a bactericidal agent (Muskhazli et al., 2009). Bakar et al. (2006) and Ismail et al. (2000) mentioned that those therapeutic activities were attributed to its mineral contents, vitamins, alkaloids, polyphenol content, as well as phytochemicals such as catechin that were reported to be in abundance in this plant. The mixture of the bioactive constituents present in *S. crispus* results in a synergistic positive effect for chronic conditions, for example, hyperglycaemia, hypertension, and cancer (Nurraihana & Norfarizan-Hanoon, 2013). *Strobilanthes crispus* leaf extract has been reported to contain high antioxidant activity attributed to various phenolic constituents, for example, catechin, caffeic acid, kaempferol, and luteolin, which contribute to its radical scavenging activity and ability to eradicate oxidative stress reactions (Al-Henhena et al., 2015; Liza et al., 2010). Antioxidant is a significant compound that acts as a health-protecting factor by lowering the risks and dangers of oxidative stress-related diseases as well as giving a health-enhancing effect on human health (Adorjan & Buchbauer, 2010), which corresponds with local lifestyle practices of using *S. crispus* as herbal tea and concoctions for increasing overall well-being (Chua et al., 2019).

Drying has become an indispensable step in processing herbal medicinal plants (Poós & Varju, 2017) because the process reduces the risk of microbial spoilage and enhances the shelf life of herbs for beneficial purposes while providing advantages such as reducing transportation and storage costs simultaneously (Barimah et al., 2017). However, drying may result in alterations in the aroma and physical characteristics of the samples with a loss of essential

phytochemicals, particularly antioxidant properties, which can affect the utmost plant quality (Orphanides et al., 2013). Various studies have reported that drying methods can have an impact on the antioxidant activity of plant products (Kuljarachanan et al., 2009; Park et al., 2006). Hajimehdipoor et al. (2012) suggested that each plant requires a specialised drying method due to differences in plant constituents in different plant species, which may result in varying levels of bioactive compounds as a result of methodological differences. After that, the ideal drying method is often debated as it becomes the earlier crucial step in preserving raw materials. It could have adverse effects by degrading the bioactive compounds and polyphenols (Chua et al., 2019). It is especially critical for heat-sensitive compounds, as their degradation may occur at specific high temperatures unique to each sample. If the drying process is unsuitable, these compounds are at risk of being lost. It is of concern when there is a considerable loss of compounds during processing, resulting in a waste of raw materials. Hence, many studies have discussed drying methods to minimise the degradation due to heat treatment so that the compounds can be optimised when extracted for various beneficial purposes. Additionally, plant species, cost, final colour, and nutritional value of dried material, as well as the time cost, should be taken into consideration when choosing the best drying method (Roshanak et al., 2016).

A previous report has compared microwave and oven drying for *S. crispus* leaves (Lasano et al., 2018) but did not address the possible difference between microwave drying and freeze drying, a method highly reported for its efficiency in preserving bioactive compounds and antioxidant properties in dried leaves (Babu et al., 2018; Thamkaew et al., 2021). Moreover, there is insufficient data on air drying for *S. crispus* compared to other methods, despite air drying being the common approach for drying herbal leaves (Babu et al., 2018).

Therefore, this study compares microwave, freeze, air, and oven drying for *S. crispus* leaves. Since *S. crispus* leaves can be a potentially useful source of many bioactive compounds, it is crucial to determine the best drying method to preserve the phytochemical and bioactive compounds in *S. crispus*. This study, therefore, may contribute to the body of knowledge in determining the efficient drying method for retaining the desirable compounds specifically for *S. crispus* leaves and become a factor in optimising the potential utilisation of this natural source in various fields to reap its benefits.

## MATERIALS AND METHODS

### Chemical Reagents

Methanol, hydrochloric acid (HCl), acetic acid ($C_2H_4O_2$), and iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) were purchased from R&M Chemicals (Malaysia). Meanwhile, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, sodium carbonate ($Na_2CO_3$), Trolox, sodium acetate

trihydrate ($NaC_2H_3O_2.3H_2O$), aluminium chloride hexahydrate ($AlCl_3.6H_2O$), gallic acid, butylated hydroxytoluene (BHT), and quercetin were obtained from Sigma Aldrich Company (USA). As for 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), it was obtained from Alfa Aesar Company (USA). All chemicals used were of analytical grade.

## Collection and Preparation of Plant Material

The *S. crispus* leaves were collected from Kampung Serdang Baru, Kuala Terengganu, Malaysia. During the collection, the leaves were separated from the stalks (Ismail et al., 2000) and transported to the laboratory. The leaves were cleaned briefly with wet tissue paper and immediately subjected to different drying methods (Lasano et al., 2018). A specimen of the plant was also deposited in a herbarium for plant identification and reference.

## Drying Methods

*Strobilanthes crispus* fresh leaves were subjected to four different drying methods, which include oven drying (OD), microwave drying (MD), freeze-drying (FD), and air drying (AD). In oven drying (OD), the leaves were arranged and spread on the trays or sheets of paper and, after that, dried in the convection oven drying chambers (Memmert, Model UN750 Plus, Germany) at 60 ± 2°C overnight (Ismail et al., 2000). In microwave drying (MD), the method was slightly modified from Lasano et al. (2018), where the leaves were spread on the provided metal rack in a standard domestic microwave (Panasonic Model, NN-GF560M, Malaysia) and dried at 900 W for 5 min. In freeze drying (FD), the fresh leaves were pre-frozen at -80ºC prior to freeze drying. The leaves were then freeze-dried in a freeze dryer (SP Scientific Virtis AdVantage 2.0 BenchTop Freeze Dryer/Lyophilizer Model Advantage Plus ES-53, USA) at a pressure of 65 Pa and a temperature of -50 ± 2°C for 4 days (Chua et al., 2019). Air drying of *S. crispus* fresh leaves was performed at 27 ± 2°C for a fortnight by arranging the leaves in thin layers on plastic sheets on an even surface such as a table and leaving them to dry in a room (Koay et al., 2013). After drying, the dried leaves were crushed instantly into pieces by an electrical dry blender (Panasonic, Model MX-SM1031, Malaysia), and lastly, ground by a rock grinder (ROCKLABS, Model 1A, New Zealand) to gain the smooth, fine powder leaves (250 µm particle size) for further extraction (Ahmed et al., 2011; Zayed et al., 2014).

## Colour Assessment of Fresh and Dried *S. crispus* Leaves

Chromameter (CR-400, Konica Minolta Sensing, Japan) analysed fresh and all dried leaves. Photographs were obtained to compare leaves that had dried by different drying methods (Chua et al., 2019). Colour differences ($\Delta E$) of colour values were calculated using Equation 1 (Tezcan et al., 2021).

$$\Delta E = \sqrt{\Delta L *^2 + \Delta a *^2 + \Delta b *^2} \quad [1]$$

where, $\Delta L^* = L^*_{\text{dried sample (2)}} - L^*_{\text{fresh/standard (1)}}$, $\Delta a^* = a^*_{\text{dried sample (2)}} - a^*_{\text{fresh/standard (1)}}$, $\Delta b^* = b^*_{\text{dried sample (2)}} - b^*_{\text{fresh/standard (1)}}$.

## Extraction of Dried *S. crispus* Leaves

*Strobilanthes crispus* powdered leaves were extracted with 80% methanol (1:10) in a sonicator (Bransonic, Model 5510R-DTH, 42 kHz ± 6%, USA) for 30 min at room temperature (27 ± 2°C) (Abas et al., 2020). The extract was then filtered with No. 2 Whatman filter paper (MonotaRO, Malaysia) with a vacuum pump (Rocker, Model Rocker 300, Taiwan) for faster filtration. The residue from the filtration was re-extracted twice with 80% methanol, which was then filtered and combined with the previous filtrate obtained. The filtrate was then concentrated under reduced pressure in a rotational evaporator (Buchi, Rotavapor® R-300, Büchi, Switzerland), and the obtained crude extracts were stored at 4°C in a chiller (Hassanbaglou et al., 2012).

## Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The analysis of TPC was carried out following Aryal et al. (2019), with some modifications. An amount of 200 µl *Strobilanthes crispus* leaves crude extract (1 mg/ml reconstituted in 80% methanol) was mixed with 1.0 ml of 0.10 M Folin-Ciocalteu's reagent in the test tube and allowed to stand for 5 min at room temperature. A volume of 1.5 ml of 7.5% sodium carbonate ($Na_2CO_3$) was added, agitated using a vortex for 30 s, and incubated at room temperature (27 ± 2°C) for 45 min. After that, the absorbance readings at 765 nm were measured using a spectrophotometer (Shimadzu, UV-1800, Japan) against a blank (80% methanol). The assay was carried out in triplicate. A standard reference calibration curve was established using gallic acid with a concentration ranging from 0.01 to 0.05 mg/ml. The TPC values were expressed as mg/g of gallic acid equivalents in milligramme per gramme (mg GAE/g) of dry extract.

TFC was assessed using an aluminium chloride colourimetric assay following Aryal et al. (2019) and Formagio et al. (2014), with a few adjustments. An aliquot of 500 µl of sample (1 mg/ml) *S. crispus* leaf extract was mixed with 1.5 ml of 95% methanol, 100 µl of 10% aluminium chloride hexahydrate ($AlCl_3.6H_2O$), 100 µl of 7.5% sodium acetate trihydrate ($NaC_2H_3O_2.3H_2O$), and 2.8 ml of distilled water. The solution in the test tubes was mixed evenly using a vortex for 30 s and kept in the dark for 40 min. After the incubation, the absorbances were taken using a spectrophotometer (Shimadzu, UV-1800, Japan) at 415 nm, and assays were carried out in triplicate. A standard quercetin calibration curve was made using the same steps as the sample extract in the 0.01–0.07 mg/ml concentration range. The TFC values were expressed as mg/g of quercetin equivalents in milligramme per gramme (mg QE/g) of dry extract.

### 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The antioxidant activity of *S. crispus* leaf extract dried with different drying methods was measured using the DPPH radical scavenging assay following Aryal et al. (2019) with slight modifications. Two (2.0) ml of *S. crispus* extract solution (10-210 µg/ml in methanol) was mixed into 2.0 ml of DPPH (0.1 mM) solution, vortexed for 15 s, and kept in the dark for 30 min. Decolourisation of DPPH-donated protons was determined by measuring the absorbance at 517 nm by a spectrophotometer (Shimadzu, UV-1800, Japan) against an equal amount of DPPH and pure methanol as a blank. The assay was carried out in triplicate. The percentage of DPPH scavenging activity was calculated using Equation 2, and the half maximal inhibitory concentration ($IC_{50}$) values for samples were determined. A sample with a smaller $IC_{50}$ value was considered to exhibit stronger antioxidant activity. The scavenging activity was compared with the synthetic antioxidant BHT.

$$\text{DPPH scavenging activity (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100\% \quad [2]$$

where, $A_0$ = absorbance of the control and $A_1$ = absorbance of the test extracts.

### Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was conducted following Benzie and Strain (1996), Iqbal et al. (2015), and Lasano et al. (2018), with slight adjustments. The FRAP assay was measured based on the rapid reduction of ferric-tripyridyltriazine (Fe (III)-TPTZ) by antioxidants present in the *S. crispus* leaf extract, forming ferrous-tripyridyltriazine (Fe (II)-TPTZ), a blue-coloured product (Payne et al., 2013). All samples were assayed in triplicate. The reagents used for stock solutions contain 300 mM acetate buffer in pH 3.6 (3.1 g sodium acetate trihydrate, $C_2H_3NaO_2 \cdot 3H_2O$ in 500 ml distilled water, and 16 ml acetic acid $C_2H_4O_2$, marked up to 1 L), 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) solution in 40 mM HCl (hydrochloric acid) and 20 mM $FeCl_3.6H_2O$ (Iron (III) chloride hexahydrate) solution. The FRAP reagent was prepared beforehand by mixing 50 ml of 300 mM acetate buffer, 5 ml of TPTZ solution, and 5 ml of 20 mM $FeCl_3.\ 6H_2O$ solution (ratio 10:1:1). The FRAP reagent was warmed at 37ºC in a water bath for 10 min. After that, 3 ml of FRAP reagent was added to the cuvette, and a blank reading was taken at 593 nm using a spectrophotometer (Shimadzu, UV-1800, Japan). Then, 100 µl of *S. crispus* leaf extract (0–125 µg/ml) mixed with 300 µl of distilled water were added together into the 3 ml of FRAP reagent in the test tubes. The mixture was then kept in the dark for 4 min, and the second absorbance reading was conducted at 593 nm. The change in the absorbance value determined the FRAP values for all samples after 4 min from the initial blank reading. The concentration of FRAP content in the extract was determined as µg Trolox equivalent (TE)/g extract basis.

### Statistical Analysis

Statistical analysis was performed using the IBM SPSS Statistics package (version 26). The data were analysed by one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) multiple comparison test for the parametric test. In contrast, as for the non-parametric test, the data were analysed using the Kruskal-Wallis H test and further determined with multiple pairwise comparisons. The values were recorded as means with a standard deviation from three replicates. The significant difference was considered significant at $p < 0.05$.

## RESULTS AND DISCUSSION

### Colour of Fresh and Dried *S. crispus* Leaves

Fresh and dried *S. crispus* leaves were assessed for their colour using physical evaluation and instrumental measurement using the chromameter. Figure 1 shows the difference in colour of the dried leaf powder obtained by different drying techniques. Leaves dried by microwave drying (MD) and freeze-drying (FD) methods showed the brightest colours, while leaf powders dried by air-drying (AD) and oven-drying (OD) methods tended to show darker colours.

Table 1 shows the data obtained from the chromameter evaluation of the dried leaf powder colour. The results from the chromameter measurement support the visual images shown in Figure 1. This instrument's colour assessment was also compared on fresh leaves. Chromameter gives the value of $L^*$, which indicates lightness with lighter (+) and darker (-) values. Based on the results, freeze-dried leaves show a significantly higher $L^*$ value (45.22 ± 2.37), which illustrates a lighter colour than the fresh leaves (35.52 ± 1.18) ($p < 0.05$). Leaves dried with FD were also found to be lighter than the leaves with other tested drying methods, which are MD (31.75 ± 1.50), AD (29.03 ± 1.60), and OD (28.33 ± 1.47) ($p < 0.05$). In contrast, the $L^*$ value of microwave-dried leaves is statistically insignificant ($p > 0.05$) compared to fresh leaves. In fact, the MD method showed no distinct difference in the $L^*$ value from the fresh leaves. On the other hand, the leaves dried with AD and OD show a significantly lower $L^*$ value ($p < 0.05$), indicating a darker colour than the fresh leaves. From these observations, FD, AD, and OD were found to have an impact on the lightness of the leaves, while MD was able to retain the fresh leaves' colour even after the drying process.

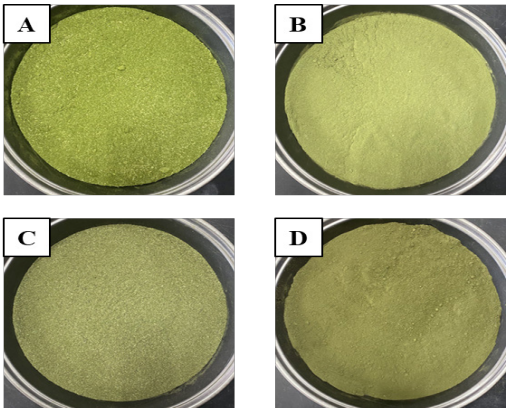



*Figure 1*. Ground dried *Strobilanthes crispus* leaves after drying by different drying methods: (A) microwave drying, (B) freeze drying, (C) air drying, (D) oven drying, respectively

Table 1
*Colour parameters L*, a*, and b* of* Strobilanthes crispus *affected by drying methods*

| Drying methods | Colour parameters | | | |
|---|---|---|---|---|
| | *L** | *a** | *b** | *ΔE* |
| Fresh leaves | 35.52 ± 1.18$^{b}$ | -12.95 ± 1.04$^{a}$ | 15.95 ± 1.54$^{b}$ | - |
| MD | 31.75 ± 1.50$^{ab}$ | -10.08 ± 1.00$^{a}$ | 14.93 ± 0.84$^{b}$ | 5.24 ±1.01$^{a}$ |
| FD | 45.22 ± 2.37$^{c}$ | -10.63 ± 0.31$^{a}$ | 17.67 ± 1.54$^{b}$ | 10.28 ± 1.38$^{b}$ |
| AD | 29.03 ± 1.60$^{a}$ | -3.36 ± 1.40$^{b}$ | 6.72 ± 1.35$^{a}$ | 14.87 ± 0.56$^{c}$ |
| OD | 28.33 ± 1.47$^{a}$ | -3.10 ± 1.53$^{b}$ | 8.99 ± 0.88$^{a}$ | 14.07 ± 0.91$^{c}$ |

*Note*. Data is represented as means ± SD (n = 3); Values followed with different superscript letters in the same column indicate significant differences at $p < 0.05$; MD = Microwave drying; FD = Freeze drying; AD = Air drying; OD = Oven drying; *L** = Lightness; *a** = Red/green coordinate; *b** = Yellow/blue coordinate; Δ*E* = Colour difference

The value *a** depicts the colour of red (+) and green (-) present in the samples, with the outcome of fresh leaves (-12.95 ± 1.04) showing the greenest colour, followed by leaves dried with FD (-10.63 ± 0.31), MD (-10.08 ± 1.00), AD (-3.36 ± 1.40), and lastly OD (-3.10 ± 1.53). Freeze-dried and microwave-dried leaves recorded statistically insignificant different values ($p > 0.05$) of *a** compared to fresh leaves. This finding manifests MD as a drying method that can retain the green colour of fresh leaves, apart from FD. Meanwhile, the *a** value for air-dried and oven-dried leaves increased considerably ($p < 0.05$) compared to fresh, microwave-dried and freeze-dried leaves, implying that the natural green of leaves dried with AD and OD underwent some losses. Meanwhile, the *b** value, which indicates yellow (+) shade in the sample, shows the highest tint of yellow in freeze-dried leaves (17.67 ± 1.54), followed by fresh leaves (15.95 ± 1.54), leaves dried with MD (14.93 ± 0.84), OD (8.99 ± 0.88), and lastly AD (6.72 ± 1.35). Freeze-dried and microwave-dried leaves have no significant difference in *b** values ($p > 0.05$) from the fresh leaves. Conversely, the *b** value for oven-dried as well as air-dried leaves reduced significantly ($p < 0.05$) in comparison to fresh leaves, suggesting that the initial yellowish tint in the leaves has been lost and there was a tendency to a darker colour on the dried leaves.

The results observed from this study are in accordance with studies by Chua et al. (2019) and Roshanak et al. (2016), where the *L** and *b** values of freeze-dried leaves were reported to show the highest value when compared to other treatments, including the fresh leaves themselves. For the *a** value, previously mentioned reports recorded the *a** value of freeze-dried leaves as being the greenest compared to the fresh leaves. However, the observation from the present results shows that freeze-dried leaves (-10.63 ± 0.31) could not be compared to the potent green colour of fresh leaves when fresh leaves recorded the highest green *a** value (-12.95 ± 1.04).

Therefore, freeze-dried leaves appeared to be lighter and yellower. Meanwhile, oven-dried leaves produced the darkest leaves among all the tested drying treatments, with a substantial loss of green pigments. It is probably caused by prolonged exposure to high drying temperatures (60°C), which may have accelerated chlorophyll degradation (Chua et al., 2019). Low $a^*$ (-) values in OD (-3.10 ± 1.53) and AD (-3.36 ± 1.40) indicated that there is a significant loss of green colour, which is associated with chlorophyll degradation as well as low retention of chlorophyll *a* (Rubinskienė et al., 2015).

Chlorophylls are unstable green pigments that are easily transformed or degraded, producing derivatives of olive-brownish, greenish, or even colourless substances (Chua et al., 2019). *Strobilanthes crispus* leaves are green herbal medicinal plants, where chlorophyll degradation is the most common occurrence during drying that may change the colour of leaves (Thamkaew et al., 2021). Drying with prolonged heat temperature led to the release of chlorophyll, a molecule from the protein complex, which could promote the transformation of chlorophylls into pheophytins (olive-brown) due to greater exposure of the chlorophylls' structure to heat (Thamkaew et al., 2021). Meanwhile, similarly to FD, MD is a drying method that retained the colour of *S. crispus* leaves without major alterations compared to fresh leaves based on the recorded $L^*$, $a^*$, and $b^*$ values.

The $\Delta E$ value, which refers to the total colour differences of dried *S. crispus* leaves in comparison to fresh leaves, was calculated and reported in Table 1. The $\Delta E$ value is vital to determining the differences, especially for comparing different tested drying methods. Previous research attested that greater $\Delta E$ values of more than 3 delineate distinct colour differences (Pathare et al., 2013; Tezcan et al., 2021). The $\Delta E$ values measured in the current study show that all tested drying methods resulted in a distinct colour difference compared to the fresh leaves (MD: 5.24 ± 1.01, FD: 10.28 ± 1.38, AD: 14.87 ± 0.56, OD: 14.07 ± 0.91). However, despite the noticeable total colour difference, *S. crispus* dried with MD recorded the lowest $\Delta E$ value, statistically different ($p < 0.05$) from the rest of the drying methods. It is indicated that microwave-dried leaves showed a similar appearance as fresh leaves, as determined by the smallest value of $\Delta E$, with insignificant differences in the data of $L^*$, $a^*$, and $b^*$ regarding the fresh leaves. These data suggest that MD can be a better and more efficient drying method to reduce colour changes from the fresh leaves. Ultimately, the modifications within colour attributes are greatly influenced by the leaves' properties and the drying methods used to carry out the process, as supported by Rubinskienė et al. (2015).

## Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) in Dried *S. crispus* Leaves

The TPC and TFC values of all dried *S. crispus* leaf extracts, expressed in mg gallic acid equivalents (GAE) and quercetin equivalents (QE) per gramme of dry extract,

are shown in Table 2. The result shows that the *S. crispus* leaves dried with MD contained the highest TPC, followed by FD, AD, and lastly OD (145.42 ± 1.61, 137.02 ± 6.90, 133.92 ± 5.29, and 19.05 ± 1.39 mg GAE/g, respectively). *Strobilanthes crispus* leaves dried by OD have a significant TPC reduction ($p < 0.05$), down to a double-digit value compared to MD, FD, and AD. Although the TPC value in microwave-dried leaves was found to have an insignificant difference ($p > 0.05$) with leaves dried with FD and AD, there was a tendency for better retention of TPC using this MD method. This occurrence coincided with Lasano et al. (2018), who recorded a better TPC value in microwave-dried leaves.

According to Chua et al. (2019), the enzymes associated with degradation in *S. crispus* may reach optimal productivity at 50°C. For example, polyphenol oxidase expedites the disintegration of phenolic compounds if activated by high-temperature drying methods, as notably occurred in OD, where the bioactive compounds are possibly degraded (Barimah et al., 2017). As a result, phenolics were most likely disintegrated when *S. crispus* leaves were subjected to temperatures beyond 50ºC for many hours in the OD. Moreover, extreme thermal processing may affect the cell microstructure, thus accelerating compound migration and further losing polyphenols (Barimah et al., 2017). Apart from that, the chemical structure of polyphenols might undergo some changes, causing the compounds to adhere to other constituents, such as proteins, resulting in difficulties in the extraction of the polyphenol compounds, thus consequently leading to low values of TPC (Barimah et al., 2017). In accordance with the TPC level in oven-dried *S. crispus* leaves, this implies that OD may not be an efficient method, or perhaps it needs some adjustments where the temperature should be lower or the drying duration should be shortened.

In terms of comparison of the TPC values with the previous reports, the MD and OD showed a great difference, where higher values of TPC were observed compared to the previous report (Lasano et al., 2018). The current study uses methanol as the extraction solvent, while Lasano et

Table 2
*Total phenolic content and total flavonoid content of dried* Strobilanthes crispus *leaves*

| Drying methods | TPC (mg GAE/g dry extract) | TFC (mg QE/g dry extract) |
|---|---|---|
| MD | $145.42 \pm 1.61^{b}$ | $117.27 \pm 5.10^{c}$ |
| FD | $137.02 \pm 6.90^{b}$ | $64.10 \pm 1.75^{b}$ |
| AD | $133.92 \pm 5.29^{b}$ | $49.95 \pm 4.58^{a}$ |
| OD | $19.05 \pm 1.39^{a}$ | $48.92 \pm 1.91^{a}$ |

*Note*. GAE = Gallic acid equivalents; QE = Quercetin equivalent; Data is represented as means ± SD (n = 3); Values followed with different superscript letters in the same column indicate significant differences at $p < 0.05$; MD = Microwave drying; FD = Freeze drying; AD = Air drying; OD = Oven drying

al. (2018) used boiling water. The drying techniques employed were also slightly different from the previous study (Lasano et al., 2018), which used OD for 10 min at a higher temperature (95–100°C) and MD for 2 min, a shorter drying time than the current study. Therefore, the differences in solvent extraction, temperature, and drying duration may explain the varied phenolic content in the *S. crispus* leaves. Furthermore, the ultrasonic extraction method used in this study may also account for the higher total phenolic contents. The same report was further noted by Nantitanon et al. (2010), where ultrasonication extraction produced a significantly high TPC as well as antioxidant activity in guava leaf extract. Through ultrasonication, a high frequency produced by the ultrasonic bath disturbs the plant cell wall structure, thus leading to increased contact between the solvent used during extraction and the extracted plant material (Nantitanon et al., 2010). Therefore, the dissolution of active compounds desired from the plant cell was enhanced, resulting in higher phytochemicals.

In terms of the TFC, *S. crispus* leaves dried with MD contained a significantly higher ($p < 0.05$) value (117.27 ± 5.10 mg QE/g) compared to the other drying methods. The TFC value was followed by leaves dried with FD (64.10 ± 1.75), AD (49.95 ± 4.58), and OD (48.92 ± 1.91). This observation has a similar trend to that of TPC discussed previously and is supported by Hayat (2020), where the TFC of microwave-dried peppermint leaves was significantly higher than that of oven-dried leaves. Apart from that, it is observed that FD is the second best at containing TFC in *S. crispus* leaves besides MD, which is significantly ($p < 0.05$) better than both AD and OD based on the TFC amounts obtained. It can be explained by the fact that FD allows the ice crystal formation, which lies inside the plant matrix, to rupture the microstructure of the plant, thereby increasing the extractability of the phytochemicals into solvent extraction without subjecting them to any heat treatment that can further degrade the phytochemicals (Bernard et al., 2014).

Lasano et al. (2018) recorded insignificant TFC between MD and OD leaves and unfermented and fermented *S. crispus* leaves, which contradicts the findings in this study. This present study recorded a significant difference in TFC value between the dried leaves of MD and OD, with higher TFC value as well as TPC results than the reported data by Lasano et al. (2018). The different temperatures, drying durations, and extraction techniques used in this study may account for the different results obtained from the previous study. The methanol solvent used in this study as an extraction solvent has been reported to be a better solvent for extracting polyphenol constituents in comparison to chloroform, ethyl acetate, and water, and it is also satisfactory for extracting flavonoids from the microstructure of cells (Yao et al., 2004). Since *S. crispus* leaves have been reported to contain abundant amounts of catechin and epicatechin (Liza et al., 2010), the methanol solvent used would contribute to the higher TFC determined from this

study, which may lead to higher antioxidant activity. Other factors contributing to the disparate values may also be the different climates and horticulture practices of *S. crispus* leaves harvested from the sampling location because catechin content in the flavonoids group varies depending on those factors (Chan et al., 2007). The mentioned factors are further supported by Ghasemzadeh et al. (2015), who reported higher flavonoid content, including catechin, in *S. crispus* leaves harvested from Kelantan in north-east Malaysia compared to other states, which are Penang and Selangor. According to Ismail et al. (2000), catechin was abundant in *S. crispus* leaf extract, and other flavonoids were present, contributing to its high antioxidant properties.

### Antioxidant Activity in Dried *S. crispus* Leaves Measured by DPPH Radical Scavenging Assay and FRAP Assay

Table 3 illustrates the antioxidant activity of dried *S. crispus* leaves assessed using the DPPH radical scavenging and FRAP assays. The ability of dried *S. crispus* leaf extracts to quench DPPH free radicals was expressed as an inhibitory concentration, $IC_{50}$, which is the half-maximal inhibitory concentration to measure the potency of extracts in inhibiting free radicals. It denotes that the smaller $IC_{50}$ value reflected the high potency of the antioxidants assessed in the extract. *S. crispus* leaves dried with MD revealed the highest potency of DPPH scavenging activity with an $IC_{50}$ value of $7.58 \pm 0.48$ µg/ml compared to the other drying methods ($p < 0.05$). *Strobilanthes crispus* leaves dried with MD also show more potency than the synthetic antioxidant BHT ($p < 0.05$), followed by FD and AD, which were also more potent than the BHT ($IC_{50}$ values of $26.59 \pm 2.78$, $42.28 \pm 0.45$, and $55.98 \pm 2.75$ µg/ml, respectively). Meanwhile, *S. crispus* leaves dried with OD showed the highest $IC_{50}$ value of $438.73 \pm 0.76$ µg/ml, indicating the weakest antioxidant potency.

The findings in this study strongly suggest that there is a good scavenging activity in *S. crispus* leaves dried using MD compared to other tested drying methods, which may be attributed to the higher preservation of phenolic and flavonoid content as observed in the analysis of TPC and TFC described earlier. Phenolic and flavonoid constituents can inhibit free radicals because their antioxidant activity is primarily due to their redox attributes, which exert an important duty of adsorbing and neutralising the free radicals, as well as quenching singlet and triplet oxygen or even decomposing peroxides (Hajimehdipoor et al., 2012; Zheng & Wang, 2001). Also, microwave-dried *S. crispus* leaves exhibited higher scavenging activity than oven-dried leaves, agreeing with Lasano et al. (2018). Furthermore, according to Lasano et al. (2018), the moisture evaporated faster during MD as a result of an elevation inside the inner temperature with a bigger vapour pressure produced, which aided the release of phytochemicals out of the samples during extraction. Based on the same trend, MD > FD > AD > OD, between TPC, TFC, and antioxidant activity determined by the DPPH radical scavenging assay, it can be

Table 3
*DPPH free radical scavenging $IC_{50}$ and ferric reducing antioxidant power of dried* Strobilanthes crispus *leaves*

| Drying methods | DPPH• scavenging $IC_{50}$ (µg/ml) | FRAP value (µg TE/g extract) |
|---|---|---|
| Standard - BHT | $55.98 \pm 2.75^{d}$ | - |
| MD | $7.58 \pm 0.48^{a}$ | $258.92 \pm 0.15^{c}$ |
| FD | $26.59 \pm 2.78^{b}$ | $256.40 \pm 0.04^{bc}$ |
| AD | $42.28 \pm 0.45^{c}$ | $256.13 \pm 0.04^{ab}$ |
| OD | $438.73 \pm 0.76^{e}$ | $84.72 \pm 0.04^{a}$ |

*Note*. Data is represented as means ± SD (n = 3); Values followed with different superscript letters in the same column indicate significant differences at $p < 0.05$; $IC_{50}$ = Half maximal inhibitory concentration; TE = Trolox equivalent; DPPH = 2,2-diphenyl-1-picrylhydrazyl; FRAP = Ferric reducing antioxidant power; BHT = Butylated hydroxytoluene; MD = Microwave drying; FD = Freeze drying; AD = Air drying; OD = Oven drying

suggested that there is a direct interaction, which has been established by previous reports (Barimah et al., 2017; Hayat, 2020; Zheng & Wang, 2001).

Regarding the FRAP values of dried *S. crispus* leaves, microwave-dried leaves show the highest value of antioxidant activity (258.92 ± 0.15 µg TE/g extract), followed by leaves dried with FD (256.40 ± 0.04), AD (256.13 ± 0.04), and OD (84.72 ± 0.04), which aligns with the observation on DPPH scavenging activity discussed earlier. It is also notable that microwave-dried leaves are significantly higher ($p < 0.05$) than oven-dried leaves. These observations coincide with a previous report that determined that green tea leaves dried via microwave demonstrated higher FRAP values than other commercial green tea samples tested (Chan et al., 2007). Lasano et al. (2018) have further acknowledged that microwave-dried, fermented, and unfermented *S. crispus* leaves have higher FRAP values than oven-dried leaves. Despite the higher value of FRAP in MD-dried leaves, the difference with freeze-dried leaves was statistically insignificant ($p > 0.05$). MD allows microwave energy to accelerate the liberation of most phenolic constituents, which reside in the plant matrix in conjugated-bound form (Hayat, 2020). Furthermore, MD also provides intense heating that elevates the inner vapour gradient as well as the temperature within the microstructure tissues and further disrupts the cell wall, causing more extraction of bioactive compounds and contributing to high antioxidant activity (Hayat, 2020). Meanwhile, FD involves low temperatures, thus avoiding the possible extreme loss of bioactive compounds, especially heat-sensitive ones (Barimah et al., 2017).

Meanwhile, the lowest FRAP value in oven-dried leaves can result from higher temperatures and thermal treatment durations for this particular drying treatment. As previously discussed, lower values of TPC and TFC were recorded in the oven-dried leaves, resulting in low antioxidant

activity. This occurrence is aligned with the suggestion by Hayat (2020), where antioxidant activity coincides with TPC as well as TFC values, which explains that antioxidant activity was at least partly due to the total phenols and flavonoids of the samples. It is supported by Franke et al. (2004), who reported that flavonoid catechin and epicatechin, apart from vitamin C present, are potent antioxidants and synergistically contribute to antioxidant activity. Apart from that, the preference selection of *S. crispus* leaves for mature old dark green leaves used in the current study might contribute to better antioxidant properties than the younger leaves at the apex, as suggested by Bakar et al. (2006). This circumstance is caused by the buildup of chemical constituents, particularly phenolics, in the physiology of old mature leaves (Bakar et al., 2006). Therefore, mature old leaves of *S. crispus* might be considered for further practices or industrial applications.

## CONCLUSION

Observing the impacts of the different drying methods on *S. crispus* leaves, it can be concluded that MD produced the closest appearance to fresh leaves of *S. crispus* without major alterations to the physical colour. Apart from that, leaves dried with MD showed the highest total phenolic content, flavonoid content, and antioxidant activity compared to the values in leaves dried with FD, AD, and OD. Despite a few reports by previous studies that claimed FD as a better method for drying a few plant species (Barimah et al., 2017; Orphanides et al., 2013; Roshanak et al., 2016), current research has proven otherwise, which is in accordance with the view of Hajimehdipoor et al. (2012), who opined that each plant requires a specialised drying method due to differences in plant constituents in different plant species. This suggestion concluded that one drying method may not be suitable for all plant species because methodological differences may result in varying phenolic content and antioxidant levels (Hajimehdipoor et al., 2012). This study illustrates the excellent antioxidant activity of *S. crispus*, particularly when dried with MD. MD can, therefore, be a better alternative for drying *S. crispus* leaves since FD is a method that requires high maintenance and high cost. A lab-scale MD was utilised in this study due to the accessibility and affordability at this stage of the research. There is indeed a potential limitation in capacity. However, the findings demonstrated that even with this lab-scale equipment, MD outperformed other methods, including FD, in retaining the antioxidant properties of the leaves. Given the cost-effectiveness of lab-scale MD compared to FD, there is a need for future research to explore industrial-scale microwave drying for large quantities of samples. Despite the limitations, this study provides a valuable foundation for further investigation into the potential benefits of microwave drying on a larger scale. This study highlights the potential of MD in optimising the retention of phytoconstituents in *S. crispus* leaves, which

thus increases its effectiveness for utilisation in herbal or nutraceutical applications and could contribute to a larger scale of *S. crispus* cultivation.

## ACKNOWLEDGEMENTS

The authors acknowledge the financial support from the Malaysian Ministry of Higher Education through the Fundamental Research Grant Scheme (FRGS/1/2019/WAB11/UMT/02/1).

## REFERENCES

Abas, M. A., Hambali, K. A., Hassin, N. H., Karim, M. F. A., Ismail, L., & Rosli, H. (2020). Antifungal activity of selected Malaysia's local medicinal plants against sick building syndrome (SBS) fungi. *Asian Journal of Plant Sciences*, *19*(3), 240-245. https://doi.org/10.3923/ajps.2020.240.245

Adorjan, B., & Buchbauer, G. (2010). Biological properties of essential oils: An updated review. *Flavour and Fragrance Journal*, *25*(6), 407–426. https://doi.org/10.1002/ffj.2024

Ahmed, F., Rahman, S., Ahmed, N., Hossain, M., Biswas, A., Sarkar, S., Banna, H., & Rahmatullah, M. (2011). Evaluation of *Neolamarckia cadamba* (Roxb.) bosser leaves extract on glucose tolerance in glucose-induced hyperglycemic mice. *African Journal of Traditional, Complementary and Alternative Medicines*, *8*(1), 79–81. https://doi.org/10.4314/ajtcam.v8i1.60549

Al-Henhena, N., Khalifa, S. A. M., Ying, R. P. Y., Hassandarvish, P., Rouhollahi, E., Al-Wajeeh, N. S., Ali, H. M., Abdulla, M. A., & El-Seedi, H. R. (2015). Chemopreventive effects of *Strobilanthes crispus* leaves extract on azoxymethane-induced aberrant crypt foci in rat colon. *Scientific Reports*, *5*, 13312. https://doi.org/10.1038/srep13312

Aryal, S., Baniya, M. K., Danekhu, K., Kunwar, P., Gurung, R., & Koirala, N. (2019). Total phenolic content, flavonoid content and antioxidant potential of wild vegetables from Western Nepal. *Plants*, *8*(4), 96. https://doi.org/10.3390/plants8040096

Babu, A. K., Kumaresan, G., Raj, V. A. A., & Velraj, R. (2018). Review of leaf drying: Mechanism and influencing parameters, drying methods, nutrient preservation, and mathematical models. *Renewable and Sustainable Energy Reviews*, *90*, 536-556. https://doi.org/10.1016/j.rser.2018.04.002

Bakar, M. F. A., Teh, A. H., Rahmat, A., Othman, F., Hashim, N., & Fakurazi, S. (2006). Antiproliferative properties and antioxidant activity of various types of *Strobilanthes crispus* tea. *International Journal of Cancer Research*, *2*(2), 152-158. https://doi.org/10.3923/ijcr.2006.152.158

Barimah, J., Yanney, P., Laryea, D., & Quarcoo, C. (2017). Effect of drying methods on phytochemicals, antioxidant activity and total phenolic content of dandelion leaves. *American Journal of Food Nutrition*, *5*(4), 136-141. https://doi.org/10.12691/ajfn-5-4-4

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry*, *239*(1), 70-76. https://doi.org/10.1006/abio.1996.0292

Bernard, D., Kwabena, A. I., Osei, O. D., Daniel, G. A., Elom, S. A., & Sandra, A. (2014). The effect of different drying methods on the phytochemicals and radical scavenging activity of Ceylon cinnamon (*Cinnamomum zeylanicum*) plant parts. *European Journal of Medicinal Plants*, *4*(11), 1324-1335. https://doi.org/10.9734/EJMP/2014/11990

Chan, E. W., Lim, Y. Y., & Chew, Y. L. (2007). Antioxidant activity of *Camellia sinensis* leaves

and tea from a lowland plantation in Malaysia. *Food Chemistry*, *102*(4), 1214-1222. https://doi.org/10.1016/j.foodchem.2006.07.009

Chua, L. Y. W., Chua, B. L., Figiel, A., Chong, C. H., Wojdyło, A., Szumny, A., & Choong, T. S. Y. (2019). Antioxidant activity, and volatile and phytosterol contents of *Strobilanthes crispus* dehydrated using conventional and vacuum microwave drying methods. *Molecules*, *24*(7), 1397. https://doi.org/10.3390/molecules24071397

Formagio, A. S. N., Volobuff, C. R. F., Santiago, M., Cardoso, C. A. L., Vieira, M. D. C., & Pereira, Z. V. (2014). Evaluation of antioxidant activity, total flavonoids, tannins and phenolic compounds in *Psychotria* leaves extracts. *Antioxidants*, *3*(4), 745-757. https://doi.org/10.3390/antiox3040745

Franke, A. A., Custer, L. J., Arakaki, C., & Murphy, S. P. (2004). Vitamin C and flavonoid levels of fruits and vegetables consumed in Hawaii. *Journal of Food Composition and Analysis*, *17*(1), 1-35. https://doi.org/10.1016/S0889-1575(03)00066-8

Ghasemzadeh, A., Jaafar, H. Z. E., & Rahmat, A. (2015). Phytochemical constituents and biological activities of different extracts of *Strobilanthes crispus* (L.) Bremek leaves grown in different locations of Malaysia. *BMC Complementary and Alternative Medicine*, *15*, 422. https://doi.org/10.1186/s12906-015-0873-3

Hajimehdipoor, H., Adib, N., Khanavi, M., Mobli, M., Amin, G. R., & Moghadam, M. H. (2012). Comparative study on the effect of different methods of drying on phenolics content and antioxidant activity of some edible plants. *International Journal of Pharmaceutical Sciences and Research*, *3*(10), 3712-3716.

Hassanbaglou, B., Hamid, A. A., Roheeyati, A. M., Saleh, N. M., Abdulamir, A., Khatib, A., & Sabu, M. C. (2012). Antioxidant activity of different extracts from leaves of *Pereskia bleo* (Cactaceae). *Journal of Medicinal Plants Research*, *6*(15), 2932-2937. https://doi.org/10.5897/JMPR11.760

Hayat, K. (2020). Impact of drying methods on the functional properties of peppermint (*Mentha piperita* L.) leaves. *Science Letters*, *8*(1), 36-42.

Iqbal, E., Salim, K. A., & Lim, L. B. (2015). Phytochemical screening, total phenolics and antioxidant activities of bark and leaves extracts of *Goniothalamus velutinus* (Airy Shaw) from Brunei Darussalam. *Journal of King Saud University - Science*, *27*(3), 224-232. https://doi.org/10.1016/j.jksus.2015.02.003

Ismail, M., Manickam, E., Danial, A. M., Rahmat, A. & Yahaya, A. (2000). Chemical composition and antioxidant activity of *Strobhilanthes crispus* leaves extract. *The Journal of Nutritional Biochemistry*, *11*(11-12), 536-542. https://doi.org/10.1016/S0955-2863(00)00108-X

Koay, Y. C., Wong, K. C., Osman, H., Eldeen, I. M. S., & Asmawi, M. Z. (2013). Chemical constituents and biological activities of *Strobilanthes crispus* L. *Records of Natural Products*, *7*(1), 59-64.

Kuljarachanan, T., Devahastin, S., & Chiewchan, N. (2009). Evolution of antioxidant compounds in lime residues during drying. *Food Chemistry*, *113*(4), 944-949. https://doi.org/10.1016/j.foodchem.2008.08.026

Lasano, N. F., Rahmat, A., Ramli, N. S., & Bakar, M. F. A. (2018). Effect of oven and microwave drying on polyphenols content and antioxidant capacity of herbal tea from *Strobilanthes crispus* leaves. *Asian Journal of Pharmaceutical and Clinical Research, 11*(6), 363-368. https://doi.org/10.22159/ajpcr.2018.v11i6.24660

Liza, M. S., Rahman, R. A., Mandana, B., Jinap, S., Rahmat, A., Zaidul, I. S. M., & Hamid, A. (2010). Supercritical carbon dioxide extraction of bioactive flavonoid from *Strobilanthes crispus* (Pecah Kaca). *Food and Bioproducts Processing*, *88*(2-3), 319-326. https://doi.org/10.1016/j.fbp.2009.02.001

Mohd Fadzelly, A. B., Asmah, R., & Fauziah, O. (2006). Effects of *Strobilanthes crispus* tea aqueous extracts on glucose and lipid profile in normal and streptozotocin-induced hyperglycemic rats. *Plant Foods for Human Nutrition*, *61*, 6-11. https://doi.org/10.1007/s11130-006-0002-z

Muskhazli, M., Dirnahayu, M., Nor Azwady A. A., Nurhafiza, Y., Nor Dalilah, E., & Che Ku Nurshaira, C. K. N. (2009). Antibacterial activity of methanolic crude extracts from selected plant against *Bacillus cereus*. *Pertanika Journal of Tropical Agricultural Science*, *32*(2), 175-183.

Nantitanon, W., Yotsawimonwat, S., & Okonogi, S. (2010). Factors influencing antioxidant activities and total phenolic content of guava leaves extract. *LWT - Food Science and Technology*, *43*(7), 1095-1103. https://doi.org/10.1016/j.lwt.2010.02.015

Nurraihana, H., & Norfarizan-Hanoon, N. A. (2013). Phytochemistry, pharmacology and toxicology properties of *Strobilanthes crispus*. *International Food Research Journal*, *20*(5), 2045-2056.

Orphanides, A., Goulas, V., & Gekas, V. (2013). Effect of drying method on the phenolic content and antioxidant capacity of spearmint. *Czech Journal of Food Sciences*, *31*(5), 509-513.

Park, Y.-S., Jung, S.-T., Kang, S.-G., Delgado-Licon, E., Ayala, A. L. M., Tapia, M. S., Martín-Belloso, O., Trakhtenberg, S., & Gorinstein, S. (2006). Drying of persimmons (*Diospyros kaki* L.) and the following changes in the studied bioactive compounds and the total radical scavenging activities. *LWT - Food Science and Technology*, *39*(7), 748-755. https://doi.org/10.1016/j.lwt.2005.05.014

Pathare, P. B., Opara, U. L., & Al-Said, F. A.-J. (2013). Colour measurement and analysis in fresh and processed foods: A review. *Food and Bioprocess Technology*, *6*, 36-60. https://doi.org/10.1007/s11947-012-0867-9

Payne, A. C., Mazzer, A., Clarkson, G. J. J., & Taylor, G. (2013). Antioxidant assays – Consistent findings from FRAP and ORAC reveal a negative impact of organic cultivation on antioxidant potential in spinach but not watercress or rocket leaves. *Food Science and Nutrition*, *1*(6), 439-444. https://doi.org/10.1002/fsn3.71

Poós, T., & Varju, E. (2017). Drying characteristics of medicinal plants. *International Review of Applied Sciences and Engineering*, *8*(1), 83-91. https://doi.org/10.1556/1848.2017.8.1.12

Roshanak, S., Rahimmalek, M., & Goli, S. A. H. (2016). Evaluation of seven different drying treatments in respect to total flavonoid, phenolic, vitamin C content, chlorophyll, antioxidant activity and color of green tea (*Camellia sinensis* or *C. assamica*) leaves. *Journal of Food Science and Technology*, *53*, 721-729. https://doi.org/10.1007/s13197-015-2030-x

Rubinskienė, M., Viškelis, P., Dambrauskienė, E., Viškelis, J., & Karklelienė, R. (2015). Effect of drying methods on the chemical composition and colour of peppermint (*Mentha × piperita* L.) leaves. *Zemdirbyste-Agriculture*, *102*(2), 223-228. https://doi.org/10.13080/z-a.2015.102.029

Tezcan, D., Sabancı, S., Cevik, M., Cokgezme, O. F., & Icier, F. (2021). Infrared drying of dill leaves: Drying characteristics, temperature distributions, performance analyses and colour changes. *Food Science and Technology International*, *27*(1), 32-45. https://doi.org/10.1177/1082013220929142

Thamkaew, G., Sjöholm, I., & Galindo, F. G. (2021). A review of drying methods for improving the quality of dried herbs. *Critical Reviews in Food Science and Nutrition*, *61*(11), 1763-1786. https://doi.org/10.1080/10408398.2020.1765309

Yao, L., Jiang, Y., Datta, N., Singanusong, R., Liu, X., Duan, J., Raymont, K., Lisle, A., & Xu, Y. (2004). HPLC analyses of flavanols and phenolic acids in the fresh young shoots of tea (*Camellia sinensis*) grown in Australia. *Food Chemistry*, *84*(2), 253-263. https://doi.org/10.1016/S0308-8146(03)00209-7

Zayed, M. Z., Ahmad, F. B., Ho, W.-S., & Pang, S.-L. (2014). GC-MS analysis of phytochemical constituents in leaves extracts of *Neolamarckia cadamba* (Rubiaceae) from Malaysia. *International Journal of Pharmacy and Pharmaceutical Sciences*, *6*(9), 123-127.

Zheng, W., & Wang, S. Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry*, *49*(11), 5165-5170. https://doi.org/10.1021/jf010697n

Pertanika J. Trop. Agri. Sci. 47 (4): 1175 - 1189 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Effect of Nutrient Solution pH on the Growth and Quality of *Lactuca sativa* Grown in a Static Hydroponic System

**Siti Samsiah Yaakup, Nursyazwani Ab Halim and Phebe Ding***

*Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

## ABSTRACT

Lettuce is an easy-to-grow and nutrient-rich leafy vegetable. It grows well using a static hydroponic system, which saves space and is easy to maintain. However, understanding pH's impact on lettuce growth in static hydroponic systems is limited. Hence, this study was conducted to determine the effect of pH nutrient solution on the growth performance and eating quality of lettuce grown in a static hydroponic system. Lettuce was grown in pH 5.2, 6.2, and 7.2 nutrient solutions. Its growth performance was collected weekly, including plant height, root length, number of leaves, leaf area, leaf chlorophyll content, total dry weight, and total moisture content. The harvested lettuce was analyzed for firmness, soluble solids concentration, titratable acidity, pH, and ascorbic acid content by the fourth week after transplanting. The plant height, root length, number of leaves, leaf area, and total dry weight of lettuce were affected by the interaction between nutrient solution pH and weeks after transplanting. By the third week after transplanting, lettuce grown in pH 6.2 was 11.12 and 18.67% taller than those grown in pH 7.2 and 5.2 nutrient solutions, respectively. By the fourth week after transplanting, the firmness of lettuce grown in pH 6.2 was significantly higher than those grown in pH 5.2 and 7.2 nutrient solutions by 2.34 and 7.32%, respectively. It is concluded that lettuce should be grown in a pH 6.2 nutrient solution when using a static hydroponic system.

*Keywords*: Ascorbic acid content, dry weight, firmness, leaf area, weeks after transplanting

ARTICLE INFO

*Article history:*
Received: 05 March 2024
Accepted: 01 April 2024
Published: 02 October 2024

DOI: https://doi.org/10.47836/pjtas.47.4.08

E-mail addresses:
sy_samsiah@upm.edu.my (Siti Samsiah Yaakup)
208418@student.upm.edu.my (Nursyazwani Ab Halim)
phebe@upm.edu.my (Phebe Ding)
*Corresponding author

## INTRODUCTION

Urbanization or migration from rural to urban locations is common in developing

ISSN: 1511-3701
e-ISSN: 2231-8542

countries. It has also occurred in Malaysia where in 2022, it was reported that 78.21% of Malaysia's total population lived in urban areas such as Kuala Lumpur, Shah Alam, Johor Bahru, Ipoh, Penang Island, and Alor Setar (Statista, 2024). It implies that rural areas are facing a reduction in the labor force to produce food, which leads to food insecurities among city dwellers. The Malaysian government has introduced the National Green Technology Policy (Ministry of Energy, Green Technology and Water, 2009) and Green Earth Program in 2005 (Jabatan Perancangan Bandar dan Desa Semenanjung Malaysia [PLANMalaysia], n.d.) under the Urbanization Program to reduce the dependency on food from rural areas. This program aims to alleviate urban agriculture with particular emphasis on cultivating, processing, and distributing food in or around the city for a better food supply system to ensure food security for the urban community.

For city dwellers, space is a constraint in the production of food. However, producing vegetables through a soilless culture of hydroponics, aquaponics, aeroponics, and vertical farming surrounding their homes is possible and has been given great attention by these communities (Muhammad & Rabu, 2015). In addition, vegetable production using soilless culture is more hygienic, consistent in height, and able to be planted at high density (Hopkinson & Harris, 2019). Among the soilless culture, the static technique is a low-cost and low-maintenance hydroponic production system that removes expenditures associated with greenhouse construction and its infrastructure (Gumisiriza et al., 2022). The system can be installed on verandas, balconies, and front or back gardens, and its materials can be reused numerous times. A nutrient solution is simply poured into a reservoir while plants are grown in the hydroponic net pots of floating polystyrene panels that float on a nutrient solution (Chang et al., 2018). The plant roots are partially or totally submerged in the nutrient solution until harvest. The nutrient solution is either replaced periodically with a fresh one or refilled after reaching a certain level of depletion (Biswas & Das, 2022). However, this system is only suitable for light feeders of leafy vegetables such as lettuce.

Lettuce or *Lactuca sativa* is widely grown worldwide and can be consumed raw or in salad. It is one of the most popular green leafy vegetables among Malaysians due to its flavor and great nutritional value (Yuen, 2023). Lettuce is low in sodium, calories, and fat but rich in iron, folate, vitamin C, and fiber (Kim et al., 2016). It is thought to be a crucial source of phytonutrients to treat inflammation, pain, gastrointestinal issues, and urinary tract infections (Noumedem et al., 2017). As such, lettuce is commonly cultivated by urban farming communities using a static hydroponic setup. However, the growth performance of lettuce often relies on readily available dissolved formulations of hydroponic fertilizers, which contain all the necessary macro- and micronutrients for optimal plant growth. However, the

solubility and availability of essential nutrients for hydroponically cultivated crops are affected by nutrient solution pH (Solis & Gabutan, 2023).

The pH of nutrient solution is known to significantly affect nutrient solubility, particularly for micronutrients such as boron, copper, iron, manganese, and zinc, influencing their availability for uptake by plant roots (Sonneveld & Voogt, 2009). A nutrient solution with a high pH (e.g., > 6.5) may lead to nutrient removal through precipitation and depletion. In contrast, a moderately low pH (e.g., 5.8) helps maintain the availability of most solution ions (Anderson et al., 2017). Kudirka et al. (2023) found that lettuce cultivated in a nutrient solution with a pH of 5.0–5.5 exhibited a 41.4% reduction in zinc accumulation in its roots compared to lettuce cultivated in the pH range of 5.5–6.0. Zinc plays a crucial role as a regulatory component in numerous plant proteins and enzymes, contributing to various cellular and physiological activities related to plant growth, development, and yield (Saleem et al., 2022). Furthermore, the pH of the nutrient solution also influences the lettuce leaf area. Lettuce grown in a pH range of 5.0–5.5 exhibited 36 and 30% smaller leaf areas compared to those cultivated in pH ranges of 5.5–6.0 and 6.0–6.5, respectively (Kudirka et al., 2023). In another study, dandelion (*Taxacum officinale*) grown in pH 4.0 nutrient solution exhibited lower marketable leaf fresh weight compared to those grown in pH 5.5 and 7.0 (Alexopoulos et al., 2021). Additionally, the plants grown at pH 4.0 had less root fresh weight per plant compared to those grown at pH 5.5. However, the study also revealed that dandelion's nutritional and dietary value increased when the crop was grown in a pH 4.0 nutrient solution compared to other pH levels. The total soluble solids, titratable acidity, total phenolics, chlorophyll *a*, chlorophyll *b*, and total chlorophylls of dandelion leaves grown in pH 4.0 nutrient solution increased by 22.9, 29.2, 26.9, 13.45, 12.8, and 13.2%, respectively as compared to those grown in pH 7.0.

Leafy vegetables are usually cultivated in nutrient solutions of pH 5.5–6.5 (Savvas & Gruda, 2018). However, the optimum range of nutrient solution pH for ideal growth differs between plant species, cultivars and environmental, substrate or nutrient solution conditions. Despite the significant impact of pH on nutrient availability to plant roots, it often receives less attention from urban agriculture communities. Instead, they take heed of nutrient solution electrical conductivity (EC). The EC provides a reliable indication of the total ion concentration, facilitating the maintenance of optimal nutrient levels for plant growth (Singh & Bruce, 2016). Nevertheless, the availability of nutrients by plant roots is affected by the pH level of the nutrient solution. Unfortunately, very little information is available on the optimal nutrient solution pH for growing lettuce in a static hydroponic setup. Hence, this study was conducted to determine the effect of the pH of the nutrient solution on growth performance and the eating quality of lettuce grown in a static hydroponic system.

## MATERIALS AND METHODS

### Lettuce Cultivation and Experimental Setup

Lettuce seeds (variety 004, Green World, Malaysia) were obtained from a local farm supply store (Dsyira Enterprise, Malaysia). A seed was sown into a sponge (2.5 x 2.5 x 2.5 $cm^3$), then placed into a plastic container (56 x 42 x 13 $cm^3$) filled with sufficient clean water. The container was covered with a dark plastic bag for three days. After three days, the bag was removed upon emergence of the lettuce radicles. Two true leaves emerged from each of the germinated seeds after 7 days. The 7-day-old healthy seedlings were transferred into 5-cm hydroponic net pots, with one plant per pot.

A total of eight 5-cm hydroponic net pots were then placed into the hole of a polystyrene panel that was set on the top of a nutrient reservoir (56 x 42 x 13 $cm^3$). The reservoirs were then filled with 18 L nutrient solutions prepared from AB Mix (Dsyira Enterprise, Malaysia). The AB Mix of hydroponic nutrition comprises A and B nutrients. Each component, weighing 12.5 kg, was simultaneously dissolved in 100 L of water using two separate tanks, creating stock solutions. The composition of stock A and B nutrient solutions is provided in Table 1.

The EC of nutrient solution in the reservoirs was maintained at 0.75, 1.0, 1.5, and 1.75 mS/cm during 0, 1, 2, and 3 weeks after transplanting, respectively (Nursyafiza, 2023). The EC levels were adjusted by adding stock solutions A and B to increase the nutrient solution EC or by reducing the water volume to lower the nutrient solution EC. An EC meter (DIST4 Hl98304, Hanna Instruments, Romania) was used to monitor the EC levels of nutrient solution weekly. The initial pH of the 18-L nutrient solution was 7.2 (after mixing 40 ml stock solution A and 40 ml solution B into water). Hence, a nutrient solution of pH 7.2 was used as

Table 1
*Composition of nutrient solution stock A and stock B that forms AB Mix (Dsyira Enterprise, Malaysia)*

| Stock A | | Stock B | |
|---|---|---|---|
| Nutrients | Quantity (g) | Nutrients | Quantity (g) |
| Calcium nitrate | 18,720 | Potassium nitrate | 16,240 |
| Iron (Ethylenediaminetetraacetic acid [EDTA]) | 328 | Magnesium sulfate | 9,840 |
| | | Monopotassium phosphate | 5,260 |
| | | Cuprum (EDTA) | 1.56 |
| | | Manganese sulfate | 30 |
| | | Boric acid | 60 |
| | | Zinc (EDTA) | 4.40 |
| | | Ammonium molybdate | 0.36 |

a control in the current study, while pH 5.2 and 6.2 were used as treatments. A 35% hydrochloric acid (Nacalai Tesque, Inc., Japan) was used to lower the nutrient solution pH to 5.2 and 6.2. The nutrient solution's pH was monitored weekly using a pH meter (HI98107, Hanna Instruments, Romania).

The study was laid in a completely randomized design with three treatments (pH 5.2, 6.2, and 7.2) and three replications. Three reservoirs of lettuce plants were prepared for each replication, leading to 72 plants per treatment, and a total of 216 plants were used in this study. The 27 reservoirs were arranged in a fully netted rain shelter with solid roofs of clear plastic. A 50% shade mesh net was used to reduce sunlight from the roof. The maximum day temperature and minimum night temperature of the rain shelter were 41 and 24.5°C, respectively. At the same time, the relative humidity of the rain shelter ranged from 49.5 to 54.0% during the day and 72.5 to 91% at night with the help of a data logger (EL-USB-2, Lascar, Hong Kong).

Since the lettuce was grown in a fully netted rain shelter, agrochemicals were not used to control pests and diseases. The depleted nutrient solution was topped up weekly to maintain a volume of 18 L in each reservoir while ensuring the nutrient solution was retained at a defined pH level. For growth performance analysis, one lettuce plant was harvested from each reservoir at weeks 1, 2, 3, and 4 after transplanting before 8.30 a.m. Nine plants per treatment were carefully placed in clear plastic bags and immediately transported to the laboratory for growth performance analysis. The growth performance of lettuce was analyzed for its shoot height, root length, leaf number, leaf chlorophyll content, leaf area, total dry weight, and total moisture content. By week 4, after transplanting, the remaining lettuces were harvested, packed in clean plastic bags, and carefully transported to the laboratory. The samples were kept in a 10±2°C chiller, and eating quality analysis was carried out within 48 hr. The eating quality, including leaf firmness, soluble solids concentration (SSC), titratable acidity (TA), pH, and ascorbic acid content, was analyzed.

## Determination of Lettuce Plant Growth Performance

The plant height of lettuce was measured from the aboveground base to the tip of the highest shoot using a ruler and expressed in centimeters (cm). The root length of lettuce was measured using a ruler from the base of the aboveground to the tip of the longest root and expressed in cm. The number of completely formed leaves was counted manually and recorded. An automatic leaf area meter (Model LI-3100, LI-COR, USA) was then used to quantify the leaf area of lettuce and expressed in $cm^2$. It was followed by collecting lettuce leaf chlorophyll with a soil plant analysis development (SPAD) meter (SPAD-502, Minolta, Japan). The leaf chlorophyll was collected by scanning leaves positioned at the top, middle and bottom of a lettuce plant, and the average of the leaf chlorophyll was expressed in

the SPAD unit. The fresh weight (FW) of a lettuce plant (including both shoot and root) was weighed using an electronic balance (FX-300i, A&D, Japan) before drying in an oven (Memmert-Schutzart DIN40050-IP20, Germany) at 50±2°C. After 72 hr, the total dry weight (DW) (including both shoot and root) of a lettuce plant was weighed, and the total moisture content (including both shoot and root) of the lettuce was calculated as follows: total moisture content (%) = (FW – DW)/FW * 100%.

## Determination of Lettuce Eating Quality Characteristics

The firmness of lettuce leaf was measured using the Instron Universal Testing Machine (5543P5995, Instron Corp., USA) fitted with a 6-mm diameter cylindrical probe and a 5-kg load cell. The leaf was penetrated with a probe to a depth of 1 mm at a crosshead speed of 20 mm/min. The force penetrating the leaf was recorded in Newton (N) with the help of Instron Merlin Software version M12-13664-EN. For SSC, 5 g of lettuce leaf was homogenized with 40 ml of distilled water. The homogenate was then filtered using cotton, and two drops of the filtrate were placed on the prism of a hand-held digital refractometer (ATAGO RX-5000, USA). The reading was then recorded as %SSC. The remaining filtrate prepared for SSC analysis was then used for TA and pH analysis. TA was determined using the acid-base titration method (Md Nor et al., 2023). The titration was performed using a 5-ml filtrate to which 2 drops of 1% phenolphthalein (Sigma-Aldrich, USA) were added. It was titrated against 0.1 N sodium dioxide (Sigma-Aldrich, USA) until a light pink color solution appeared. The results were expressed as a percentage of citric acid. The pH of lettuce filtrate was measured with the help of a pH meter (Crison Micro pH 2000, Crison Instruments, Spain). A modified method from Mariani et al. (2018) was used to determine the ascorbic acid content in lettuce. Five grams of lettuce leaves with their stems were homogenized with 45 ml of 2% metaphosphoric acid ($HPO_3$, Sigma-Aldrich, USA). After filtering the homogenate through cotton, 5 ml of filtrate was added to 10 ml of 2,6-dichlorophenol-indophenol dye solution (Sigma-Aldrich, USA). The mixture was then measured at a wavelength of 518 nm using a spectrophotometer (WPA S1200, SpectraWAVE, USA). The concentration of ascorbic acid in the lettuce was determined from the standard curve, and the amount of ascorbic acid in the lettuce was calculated using the following equation: ascorbic acid (mg/100 g) = (ascorbic acid content × volume made up × 100) / (volume of solution taken for estimation × 1000 × weight of sample taken).

## Statistical Analysis

The collected data were analyzed using analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was applied to separate the means when *F*-values showed significance at the 5% level, using Statistical Analysis System software (version 9.4). A two-factor ANOVA was utilized to assess

growth performance, while a single-factor analysis was employed to evaluate the eating quality of lettuce.

## RESULTS

### Growth Performance

The results of the ANOVA showed a significant interaction between the pH levels of the nutrient solution of the static hydroponic system and the weeks after transplanting (Table 2). This interaction affects various aspects of lettuce growth, including plant height, root length, number of leaves, leaf area, and total dry weight.

Figure 1 shows the interaction effects of nutrient solution pH and weeks after transplanting on lettuce plant height. Regardless of the pH levels, there was no significant difference in plant height during the first and second weeks of growing. By week 3, after transplanting, the plant height of lettuce grown in a static hydroponic system with pH 6.2 was higher than the other two pH levels. A more obvious increase in height was observed when lettuce grew to week 4 after transplanting. The height of lettuce grown in nutrient solution pH 6.2 was the highest, followed by lettuce grown in pH 7.2 and 5.2.

The root length of lettuce follows the trend of its height, where the nutrient solution pH did not affect the root length during the initial stage of growth (Figure 2). By week 3, after transplanting, the root lengths of lettuce grown at pH 6.2 and 7.2

Table 2
*Main and interaction effects of nutrient solution pH and weeks after transplanting on growth characteristics of lettuce grown in a static hydroponic system*

| Factors | Plant height (cm) | Root length (cm) | No. of leaves | Leaf chlorophyll (SPAD unit) | Leaf area ($cm^2$) | Total dry weight (g) | Total moisture content (%) |
|---|---|---|---|---|---|---|---|
| Levels of pH (L) | | | | | | | |
| 5.2 | 10.21 c[z] | 9.57 c | 10.53 b | 21.07 a | 288.41 b | 0.81 b | 94.77 a |
| 6.2 | 12.32 a | 13.67 a | 12.22 a | 20.59 a | 552.18 a | 1.41 a | 94.85 a |
| 7.2 | 10.85 b | 12.15 b | 10.13 b | 20.68 a | 321.67 b | 0.82 b | 93.88 b |
| Weeks after transplanting (W) | | | | | | | |
| 1 | 2.40 d | 5.67 c | 3.00 d | 12.38 d | 0.94 c | 0.003 c | 93.24 b |
| 2 | 4.19 c | 4.87 c | 5.00 c | 16.01 c | 5.96 c | 0.021 c | 93.73 b |
| 3 | 12.56 b | 16.60 b | 13.26 b | 28.19 a | 225.79 b | 0.571 b | 94.61 a |
| 4 | 25.16 a | 19.83 a | 22.26 a | 25.93 b | 1282.84 a | 3.365 a | 95.28 a |
| Interaction | | | | | | | |
| L x W | * | * | * | NS | * | * | NS |

*Note*. [z]Means separation (n = 9) followed by the same letters is significantly different at $P \leq 0.05$ according to Duncan's multiple range test
NS, * = Nonsignificant or highly significant at $P \leq 0.05$, respectively; SPAD = Soil plant analysis development

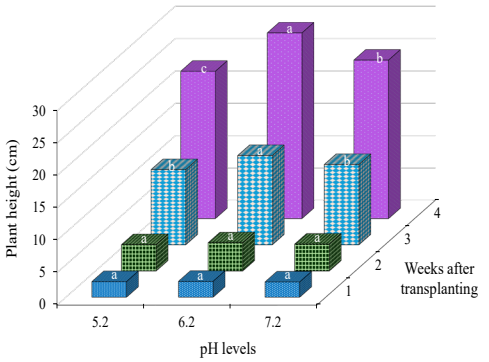



*Figure 1*. The effects of nutrient solution pH levels and weeks after transplanting on the plant height of lettuce grown in a static hydroponic system

*Note*. Means followed by different letters are significantly different ($P \leq 0.05$) within each pH level

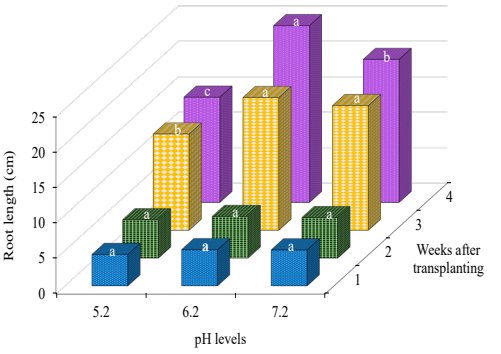



*Figure 2*. The effects of nutrient solution pH levels and weeks after transplanting on the root length of lettuce grown in a static hydroponic system

*Note*. Means followed by different letters are significantly different ($P \leq 0.05$) within each pH level

were significantly longer than those grown at pH 5.2. However, by week 4, the root length of lettuce grown in nutrient solution pH 6.2 surpassed those grown in pH 7.2 and 5.2.

The number of lettuce leaves was not affected by weeks after transplanting until it was ready to be harvested by week 4 (Figure 3). Lettuce grown in nutrient solution with a pH of 6.2 had the most leaves, followed by plants grown at pH 5.2 and 7.2. Similar to the number of leaves, there were no changes in the leaf area of lettuce that grew in nutrient solution pH 5.2, 6.2, and 7.2 during the initial stage of growth (Figure 4). By week 3, the leaf area of lettuce grown in nutrient solution pH 6.2 had surpassed other pH levels, and the trend continued until the lettuce was harvested at week 4 after transplanting.

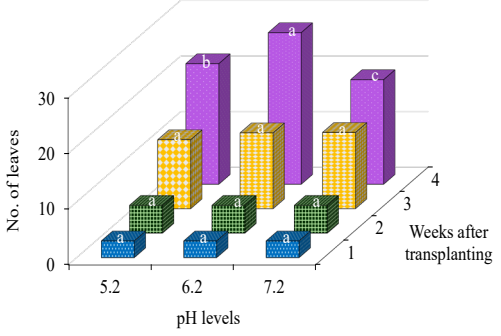



*Figure 3*. The effects of nutrient solution pH levels and weeks after transplanting on the leaf number of lettuce grown in a static hydroponic system

*Note*. Means followed by different letters are significantly different ($P \leq 0.05$) within each pH level

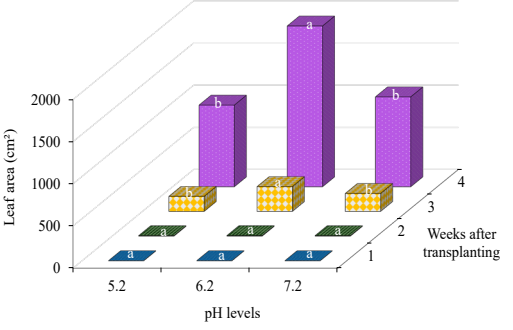



*Figure 4*. The effects of nutrient solution pH levels and weeks after transplanting on the leaf area of lettuce grown in a static hydroponic system

*Note*. Means followed by different letters are significantly different ($P \leq 0.05$) within each pH level

As found in plant height, root length, number of leaves and leaf area, the dry weight of lettuce during the first two weeks of growth did not show any differences among nutrient solutions with various pH levels (Figure 5). The differences can be found after the lettuce has grown for three weeks. When lettuce grows in nutrient solution pH 6.2, it shows a higher dry weight than other pH levels. The significant differences extended to week 4 after transplanting.

The ANOVA results show no significant interaction between the pH of the nutrient solution and the weeks after transplanting on leaf chlorophyll content and total moisture content (Table 2). Unlike other growth parameters, the chlorophyll content of lettuce was not affected by the pH of the nutrient solution. However, as measured using SPAD, the leaf chlorophyll content increased as the lettuce grew, reaching its maximum at 3 weeks after transplanting. After that, the leaf chlorophyll decreased as the lettuce approached harvest readiness. It implies that lettuce enters the senescence phase by week 4 after transplanting, which is marked by the deterioration of leaf chlorophyll pigment. For total moisture content, lettuce grown in a nutrient solution of pH 7.2 has significantly lower moisture content than those grown in pH 5.2 and 6.2 (Table 2). During initial growth, there was no change in lettuce moisture content. However, the moisture content increased by the third week after transplanting and plateaued throughout its growth.

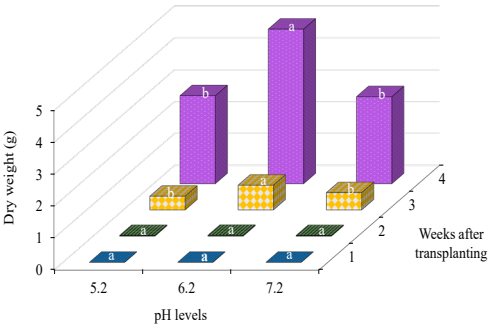



*Figure 5*. The effects of nutrient solution pH levels and weeks after transplanting on the dry weight of lettuce grown in a static hydroponic system

*Note*. Means followed by different letters are significantly different ($P \leq 0.05$) within each pH level

## Eating Quality

The eating quality of lettuce was determined immediately after harvesting. The firmness of lettuce was influenced by the pH levels of the nutrient solution during planting, with pH 6.2 yielding the firmest lettuce, followed by pH 5.2 and 7.2 (Table 3). Similarly, the concentration of soluble solids in lettuce produced from pH 6.2 nutrient solution was significantly higher than those from pH 5.2 and 7.2. However, the pH levels of the nutrient solution did not affect the TA of lettuce. Furthermore, the pH of lettuce was influenced by the pH of the nutrient solution, with lettuce grown in a system with pH 6.2 exhibiting a significantly higher pH than those grown in pH 5.2 and 7.2 solutions. Interestingly, the ascorbic acid content of lettuce did not follow the trend observed in other eating quality characteristics, as lettuce harvested from pH 6.2 nutrient solution had lower ascorbic acid content than those grown in pH 5.2.

Table 3
*Eating quality characteristics of lettuce grown in a static hydroponic system using three levels of nutrient solution pH*

| Nutrient solution pH levels | Firmness (N) | Soluble solids concentration (%SSC) | Titratable acidity (%) | pH | Ascorbic acid content (mg/100 g) |
|---|---|---|---|---|---|
| 5.2 | 57.33 b[z] | 0.63 b | 0.31 a | 6.13 b | 20.68 a |
| 6.2 | 58.67 a | 0.87 a | 0.32 a | 6.24 a | 20.00 b |
| 7.2 | 54.67 c | 0.68 b | 0.27 a | 6.17 b | 20.16 ab |

*Note*. [z]Means separation (n = 9) followed by the same letters within a column is significantly different at $P \leq 0.05$ according to Duncan's multiple range test

## DISCUSSION

The optimal pH range for most hydroponically grown leafy vegetables ranges between pH 5.5 and 6.5 (Savvas & Gruda, 2018). The current study reveals that the growth performance of lettuce grown in pH 6.2 nutrient solution is superior to those cultivated in pH 5.2 and 7.2. A similar finding was also reported in lettuce grown using the nutrient-film technique, where the performance of lettuce grown in pH 6.0 and 6.2 nutrient solutions was better than those grown in pH 5.8 and 6.4 (Samarakoon et al., 2020). *Taraxacum officinale* and *Reichardia picroides* plants grown in pH 5.5 nutrient solution using a floating hydroponic system exhibited better growth performance than those grown in pH 4.0 and 7.0 solutions (Alexopoulos et al., 2021). For 'Corvair' spinach grown in pH 4.0, 4.5, 5.0, and 5.5 nutrient solutions, shoot, and root mass decreased with decreasing nutrient solution pH, with lower pH levels producing lighter plants (Gillespie et al., 2021). The stunted growth of spinach cultivated in a pH 4.0 nutrient solution severely inhibited root development. Nutrient solution pH plays an important role in crop growth.

The significant reduction in lettuce height (Figure 1) and root length (Figure 2) grown in this study's 5.2 nutrient solution is likely due to reduced nutrient uptake by plants. Previous studies have highlighted that nutrient solution pH is one of the most critical factors affecting nutrient availability, uptake, and solubility by plants (Fathidarehnijeh et al., 2023). When pH deviates from the optimal range, nutrients become less available, rendering plants unable to absorb them efficiently. Under high pH conditions, calcium and magnesium precipitate, while iron and phosphate remain insoluble. As a result, these ions become unavailable for root absorption, leading to the inhibition of micronutrient absorption such as iron, copper, zinc, and manganese (Gillespie et al., 2020; Singh et al., 2019; Velazquez-Gonzalez et al., 2022). By analyzing spinach tissue grown in pH 4.0, 4.5, 5.0, and 5.5 nutrient solutions, it was observed that the concentration of nitrogen, phosphorus, potassium, magnesium, copper, iron, manganese, and zinc decreased as the pH of the nutrient solution decreased (Gillespie et al., 2021). Samarakoon et al. (2020) reported that lettuce grown in a

pH 6.0 nutrient solution exhibited higher nitrogen levels in its tissue, resulting in a greater yield compared to lettuce grown in a pH 5.8 nutrient solution.

Nitrogen is an important element in chlorophyll pigment and is essential for photosynthesis. In the present study, SPAD was used to measure the chlorophyll content of lettuce, and the finding reveals that the pH of the nutrient solution did not affect lettuce leaf chlorophyll content (Table 2). A similar result was reported by Singh et al. (2019), where no significant difference was observed in Swiss chard (*Beta vulgaris* L.) SPAD values among different pH levels of nutrient solutions. The SPAD meter is recognized as a useful tool for measuring leaf greenness, as it is closely linked to chlorophyll content (Azia & Stewart, 2001). The chlorophyll content in leaves is known to reflect the physiological status of a plant (Gitelson et al., 2003). It is commonly used to indicate chloroplast development, photosynthetic capacity, leaf nitrogen content, or overall plant health (Ling et al., 2011). Previous studies have reported that the SPAD value can estimate the nitrogen status of various plant species (Colla et al., 2010). This finding corroborates that the pH of nutrient solutions did not affect nitrogen availability in the lettuce of the current study. Although the chlorophyll content of lettuce in this study was not affected by the pH of the nutrient solution, it exhibited an increase from weeks 1 to 3 after transplanting, followed by a subsequent decrease (Table 2). The decline in chlorophyll content in lettuce leaves indicates the onset of senescence. The primary morphological sign of leaf senescence is the transition of leaf colour from green to yellow (Zhao et al., 2022). This phenomenon is attributed to nitrogen redistributing during leaf senescence, leading to leaf yellowing (Havé et al., 2017).

Nutrient content in the growing medium affects the eating quality of vegetables. Nitrogen application rate can affect the head compactness of crisphead varieties of lettuce, and the head weight of cos lettuce cultivated in an automated glasshouse (Konstantopoulou et al., 2010). Fresh-cut celery (*Apium graveolens*) quality was improved when grown under low or adequate nitrogen fertilization (Babalar et al., 2022). Increasing nitrogen levels decreases the vitamin C content of cauliflower (*Brassica oleracea* Botrytis Group) (Lee & Kader, 2000). In another study, increased nitrogen fertilizer rate decreased harvested cabbage's vitamin C and dietary fiber (*B. oleracea* Capitata Group). However, a contradicting finding was reported in minimally processed Swiss chard, where plants treated with higher nitrogen content contained higher vitamin C levels compared to those grown with lower fertilizer rates (Miceli et al., 2018). The nitrogen fertilization rates affect the eating quality of vegetables.

In addition to nitrogen, it was found that high rates of phosphorus fertilizer increase the SSC of tomatoes (Weston & Barth, 1997). If there is a lack of phosphorus, the filling of corn ears (*Zea mays* L.) will be affected, leading to poor-quality ears. In hydroponically grown lettuce, an increase in nutrient solution pH elevates

the proportion of potassium compared to magnesium or calcium. Insufficient calcium causes tip burn in leafy crops, blossom-end rot in tomatoes, and blackheart in celery during vegetable cultivation. These insights underscore the importance of nutrient management strategies tailored to specific crop requirements to optimize yield and quality in agricultural production. Unfortunately, the tissue nutrients of lettuce leaves were not analyzed in this study to correlate the pH of the solution with the nutrient contents in lettuce.

## CONCLUSION

The pH of the nutrient solution in a static hydroponic system can significantly impact lettuce growth, including plant height, root length, number of leaves, leaf area, and weight, particularly after 3 weeks of transplanting. Additionally, the pH of the nutrient solution influences the firmness, SSC, TA, pH, and ascorbic acid content of lettuce. In conclusion, maintaining a pH of 6.2 in a static hydroponic setup is recommended to achieve optimal growth performance and eating quality of lettuce.

## ACKNOWLEDGEMENTS

The authors acknowledged the Universiti Putra Malaysia for providing experiment facilities.

## REFERENCES

Alexopoulos, A. A., Marandos, E., Assimakopoulou, A., Vidalis, N., Petropoulos, S. A., & Karapanos, I. C. (2021). Effect of nutrient solution pH on the growth, yield and quality of *Taraxacum officinale* and *Reichardia picroides* in a floating hydroponic system. *Agronomy*, *11*(6), 1118. https://doi.org/10.3390/agronomy11061118

Anderson, T. S., Martini, M. R., Villiers, D. D., & Timmons, M. B. (2017). Growth and tissue elemental composition response of butterhead lettuce (*Lactuca sativa*, cv Flandria) to hydroponic conditions at different pH and alkalinity. *Horticulturae*, *3*(3), 41. https://doi.org/10.3390/horticulturae3030041

Azia, F., & Stewart, K. A. (2001). Relationships between extractable chlorophyll and SPAD values in muskmelon leaves. *Journal of Plant Nutrition*, *24*(6), 961-966. https://doi.org/10.1081/PLN-100103784

Babalar, M., Daneshvar, H., Díaz-Pérez, J. C., Nambeesan, S., Tabrizi, L., & Delshad, M. (2022). Effects of organic and chemical nitrogen fertilization and postharvest treatments on the visual and nutritional quality of fresh-cut celery (*Apium graveolens* L.) during storage. *Food Science and Nutrition*, *11*(1), 320-333. https://doi.org/10.1002/fsn3.3063

Biswas, S., & Das, R. (2022). Hydroponics: A promising modern intervention in agriculture. *Agriculture and Food: E-Newsletter*, *4*(1), 334-338.

Chang, C.-L., Hong, G.-F., & Fu, W.-L. (2018). Design and implementation of a knowledge-based nutrient solution irrigation system for hydroponic applications. *Transactions of the American Society of Agricultural and Biological Engineers*, *61*(2), 369-379.

Colla, G., Suarez, C. M. C., Cardarelli, M., & Rouphael, Y. (2010). Improving nitrogen use efficiency in melon by grafting. *HortScience*, *45*(4), 559-565. https://doi.org/10.21273/HORTSCI.45.4.559

Fathidarehnijeh, E., Nadeem, M., Cheema, M., Thomas, R., Krishnapillai, M., & Galagedar, L. (2023). Current perspective on nutrient solution

management strategies to improve the nutrient and water use efficiency in hydroponic systems. *Canadian Journal of Plant Science*, *104*(2), 88-102. https://doi.org/10.1139/cjps-2023-0034

Gillespie, D. P., Kubota, C., & Miller, S. A. (2020). Effects of low pH of hydroponic nutrient solution on plant growth, nutrient uptake, and root rot disease incidence of basil (*Ocimum basilicum* L.). *HortScience*, *55*(8), 1251–1258. https://doi.org/10.21273/hortsci14986-20

Gillespie, D. P., Papio, G., & Kubota, C. (2021). High nutrient concentrations of hydroponic can improve growth and nutrient uptake of spinach (*Spinacia oleracea* L.) grown in acidic nutrient solution. *HortScience*, *56*(6), 687-694. https://doi.org/10.21273/HORTSCI15777-21

Gitelson, A. A., Grits, Y., & Merzlyak, M. N. (2003). Relationships between leaf chlorophyll content and spectral reflectance and algorithms for non-destructive chlorophyll assessment in higher plant leaves. *Journal of Plant Physiology*, *160*(3), 271-282. https://doi.org/10.1078/0176-1617-00887

Gumisiriza, M. S., Ndakidemi, P., Nalunga, A., & Mbega, E. R. (2022). Building sustainable societies through vertical soilless farming: A cost-effectiveness analysis on a small-scale non-greenhouse hydroponic system. *Sustainable Cities and Society*, *83*, 103923. https://doi.org/10.1016/j.scs.2022.103923

Havé, M., Marmagne, A., Chardon, F., & Masclaux-Daubresse, C. (2017). Nitrogen remobilisation during leaf senescence: Lessons from *Arabidopsis* to crops. *Journal of Experimental Botany*, *68*(10), 2513-2529. https://doi.org/10.1093/jxb/erw365

Hopkinson, S., & Harris, M. (2019). Effect of pH on hydroponically grown bush beans (*Phaseolus vulgaris*). *International Journal of Environment Agriculture and Biotechnology*, *4*(1), 142-145. https://doi.org/10.22161/ijeab/4.1.23

Jabatan Perancangan Bandar dan Desa Semenanjung Malaysia. (n.d.). *Panduan pelaksanaan inisiatif pembangunan kejiranan hijau: Pembangunan kebun kejiranan* [Green neighbourhood development initiative implementation guide: Neighbourhood garden development]. PLANMalaysia. https://mytownnet.planmalaysia.gov.my/wp-content/uploads/2023/03/LAPORAN-PANDUAN-PELAKSANAAN-KEBUN-KEJIRANANlow.pdf

Kim, M. J., Moon, Y., Tou, J. C., Mou, B., & Waterland, N. L. (2016). Nutritional value, bioactive compounds and health benefits of lettuce (*Lactuca sativa* L.). *Journal of Food Composition and Analysis*, *49*, 19-34. https://doi.org/10.1016/j.jfca.2016.03.004

Konstantopoulou, E., Kapotis, G., Salachas, G., Petropoulos, S. A., Karapanos, I. C., & Passam, H. C. (2010). Nutritional quality of greenhouse lettuce at harvest and after storage in relation to N application and cultivation season. *Scientia Horticulturae*, *125*(2), 93.e1-93.e5. https://doi.org/10.1016/j.scienta.2010.03.003

Kudirka, G., Viršilė, A., Sutulienė, R., Laužikė, K., & Samuolienė, G. (2023). Precise management of hydroponic nutrient solution pH: The effects of minor pH changes and MES buffer molarity on lettuce physiological properties. *Horticulturae*, *9*(7), 837. https://doi.org/10.3390/horticulturae9070837

Lee, S. K., & Kader, A. A. (2000). Preharvest and postharvest factors influencing vitamin C content of horticultural crops. *Postharvest Biology and Technology*, *20*(3), 207-220. https://doi.org/10.1016/S0925-5214(00)00133-2

Ling, Q., Huang, W., & Jarvis, P. (2011). Use of SPAD-502 meter to measure leaf chlorophyll concentration in *Arabidopsis thaliana*. *Photosynthesis Research*, *107*, 209–214. https://doi.org/10.1007/s11120-010-9606-0

Mariani, M., Cahaya, N. M., & Ding, P. (2018). Physicochemical characteristics of *Carissa congesta* fruit during maturation. *Acta Horticulturae*, *1213*, 461-464. https://doi.org/10.17660/ActaHortic.2018.1213.68

Md Nor, S., Ding, P., & Tan, J. C. (2023). Locule position and thawing duration affect postharvest quality of freshly cryo-frozen Musang King Durian fruit. *Pertanika Journal of Tropical Agricultural Science*, *46*(2), 517-528. https://doi.org/10.47836/pjtas.46.2.09

Miceli, A., Mineo, V., & Planeta, D. (2018). Effect of nitrogen fertilizer level on quality and shelf-life of fresh cut Swiss chard. *Acta Horticulturae*, *1209*, 271-276. https://doi.org/10.17660/ActaHortic.2018.1209.39

Ministry of Energy, Green Technology and Water. (2009). *National Green Technology Policy* (1st ed.). National Library of Malaysia.

Muhammad, R. M., & Rabu, M. R. (2015). *The potential of urban farming technology in Malaysia: Policy intervention*. Food and Fertilizer Technology Center for the Asian and Pacific Region Agricultural Policy Platform. https://ap.fftc.org.tw/article/965

Noumedem, J. A. K., Djeussi, D. E., Hritcu, L., Mihasan, M., & Kuete, V. (2017). *Lactuca sativa*. In V. Kuete (Ed.), *Medicinal spices and vegetables from Africa: Therapeutic potential against metabolic, inflammatory, infectious and systemic diseases* (pp. 437-449). Academic Press. https://doi.org/10.1016/B978-0-12-809286-6.00020-0

Nursyafiza, M. (2023). *Effects of nutrient solution's electrical conductivity rates on lettuce (*Lactuca sativa *L.) performance under static hydroponic cultivation* [Unpublished Bachelor dissertation]. Universiti Putra Malaysia.

Saleem, H. M., Usman, K., Rizwan, M., Al Jabri, H., & Alsafran, M. (2022). Functions and strategies for enhancing zinc availability in plants for sustainable agriculture. *Frontiers in Plant Science*, *13*, 1033092. https://doi.org/10.3389/fpls.2022.1033092

Samarakoon, U., Palmer, J., Ling, P., & Altland, J. (2020). Effects of electrical conductivity, pH, and foliar application of calcium chloride on yield and tipburn of *Lactuca sativa* grown using the nutrient–film technique. *HortScience*, *55*(8), 1265-1271. https://doi.org/10.21273/HORTSCI15070-20

Savvas, D., & Gruda, N. (2018). Application of soilless culture technologies in the modern greenhouse industry – A review. *European Journal of Horticultural Science*, *83*(5), 280-293. https://doi.org/10.17660/eJHS.2018/83.5.2

Singh, H., & Bruce, D. (2016). *Electrical conductivity and pH guides for hydroponics*. Oklahoma Cooperative Extension Service. https://extension.okstate.edu/fact-sheets/print-publications/hla/electrical-conductivity-and-ph-guide-for-hydroponics-hla-6722.pdf

Singh, H., Dunn, B. L., Payton, M., & Brandenberger, L. (2019). Selection of fertilizer and cultivar of sweet pepper and eggplant for hydroponic production. *Agronomy*, *9*(8), 433. https://doi.org/10.3390/agronomy9080433

Solis, E. S., & Gabutan, J. U. (2023). Hydroponic lettuce (*Lactuca sativa* L. var. Lalique) production using commercially available nutrient solutions. *International Journal of Agriculture and Environmental Research*, *9*(3), 330-341. https://doi.org/10.51193/IJAER.2023.9306

Sonneveld, C., & Voogt, W. (2009). Plant nutrition in future greenhouse production. In *Plant nutrition of greenhouse crops* (pp. 393-403). Springer. https://doi.org/10.1007/978-90-481-2532-6_17

Statista. (2024). *Malaysia: Urbanization from 2013 to 2023*. https://www.statista.com/statistics/455880/urbanization-in-malaysia/

Velazquez-Gonzalez, R. S., Garcia-Garcia, A. L., Ventura-Zapata, E., Barceinas-Sanchez, J. D. O., & Sosa-Savedra, J. C. (2022). A review on hydroponics and the technologies associated for medium- and small-scale operations. *Agriculture*, *12*(5), 646. https://doi.org/10.3390/agriculture12050646

Weston, L. A., & Barth, M. M. (1997). Preharvest factors affecting postharvest quality of vegetables. *HortScience*, *32*(5), 812-816. https://doi.org/10.21273/HORTSCI.32.5.812

Yuen, M. K. (2023, August 20). Interactive: Malaysia's thriving fruits and vegetables. *The Star*. https://www.thestar.com.my/news/nation/2023/08/20/interactive-malaysias-thriving-fruits-and-vegetables

Zhao, W., Zhao, H., Wang, H., & He, Y. (2022). Research progress on the relationship between leaf senescence and quality, yield and stress resistance in horticultural plants. *Frontiers in Plant Science*, *13*, 1044500. https://doi.org/10.3389/fpls.2022.1044500

Pertanika J. Trop. Agri. Sci. 47 (4): 1191 - 1204 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Optimizing Growth of Melon (*Cucumis melo* L. cv. Madesta) in Nutrient Film Technique and Drip Irrigation Hydroponics with Varied Substrates

**Yosephine Sri Wulan Manuhara[1], Djarot Sugiarso[2], Ariyan Pratama Fajar[1], Khoirul Niam[1], Raden Thilawatil Aziz[1], Arga Wal Yudha[1], Christopher Clement[1], Budi Setiadi Daryono[3], Miftahudin[4], Karlia Meitha[5], Awik Puji Dyah Nurhayati[6] and Anjar Tri Wibowo[1]***

*[1]Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Kampus C, Mulyorejo, Surabaya 60115, Indonesia*
*[2]Department of Chemistry, Fakultas Sains dan Analitika Data, Institut Teknologi Sepuluh, Jl. Raya ITS, Keputih, Surabaya 60115, Indonesia*
*[3]Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada, Jl. Teknika Selatan, Sinduadi, Yogyakarta 55281, Indonesia*
*[4]Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor. Jl. Agatis, Kampus IPB Dramaga, Bogor 16680, Indonesia*
*[5]School of Life Sciences and Technology, Bandung Institute of Technology, Jl. Ganeca No.10, Bandung 40132, Indonesia*
*[6]Department of Biology, Fakultas Sains dan Analitika Data, Institut Teknologi Sepuluh, Jl. Raya ITS, Keputih, Surabaya 60115, Indonesia*

ARTICLE INFO

*Article history:*
Received: 16 March 2024
Accepted: 05 April 2024
Published: 25 October 2024

DOI: https://doi.org/10.47836/pjtas.47.4.09

E-mail addresses:
yosephine-s-w-m@fst.unair.ac.id (Yosephine Sri Wulan Manuhara)
djarot@chem.its.ac.id (Djarot Sugiarso)
ariyan.pratama.fajar-2018@fst.unair.ac.id (Ariyan Pratama Fajar)
khoirul.niam-2018@fst.unair.ac.id (Khoirul Niam)
raden.thilawatil.aziz-2018@fst.unair.ac.id (Raden Thilawatil Aziz)
arga.wal.yudha-2018@fst.unair.ac.id (Arga Wal Yudha)
christopher.clement-2023@fst.unair.ac.id (Christopher Clement)
bs_daryono@mail.ugm.ac.id (Budi Setidadi Daryono)
miftahudin@apps.ipb.ac.id (Miftahudan)
karliameitha@sith.itb.ac.id (Karlia Meitha)
awik@bio.its.ac.id (Awik Puji Dyah Nurhayati)
anjar.tri@fst.unair.ac.id (Anjar Tri Wibowo)
*Corresponding author

## ABSTRACT

Hydroponic systems offer a promising solution for urban farming and the utilization of unproductive land. Successful implementation, however, requires careful optimization to select the most effective hydroponic system tailored to specific plants and environmental conditions. This study aims to compare the growth and physiological responses of Madesta melons (*Cucumis melo* L. cv. Madesta) cultivated using the nutrient film technique (NFT) and drip irrigation system (DIS) with variations in growth media. The Madesta melon seeds underwent a two-week germination phase in coco peat media, followed by transplanting

ISSN: 1511-3701
e-ISSN: 2231-8542

into NFT and DIS setups utilizing diverse growth media, including rice husk, rice husk mixed with compost, and compost only. Over four weeks post-cultivation, assessments were conducted on key growth metrics such as leaf count, leaf diameter, plant height, and stem diameter. Plant physiological responses were also analyzed, encompassing chlorophyll and nitrogen levels, along with the mineral composition within leaves and fruits. Results revealed that the DIS cultivation outperformed the NFT in terms of growth outcomes. Among the varied media combinations, the rice husk and compost blend supported growth most effectively. Notably, no significant differences were observed in leaf and fruit nitrogen content between the DIS and NFT systems, and the overall mineral content of the media remained relatively stable before and after the cultivation period. Mineral content analysis revealed calcium as the predominant element in the leaves, while potassium emerged as the most abundant mineral in the fruits. This research sheds light on the potential of hydroponic systems, specifically the DIS method, for enhancing melon cultivation, emphasizing the importance of selecting appropriate growth media to maximize plant growth.

*Keywords*: Agricultural innovation, drip irrigation system, hydroponic, melon, nutrient film technique

## INTRODUCTION

Rapid industrialization caused the reduction of cultivable land and the degradation of farming land due to water pollution from industrial waste and excessive fertilization. A hydroponic system is an excellent solution to address both water conservation and over-fertilization issues. Hydroponics is a method of growing plants without soil, using a nutrient-rich water solution to deliver essential minerals directly to the plant roots. This system offers several benefits, including water efficiency, precise nutrient delivery, space efficiency, faster growth, and higher yields compared to traditional methods (Velazquez-Gonzalez et al., 2022). Several hydroponic farming methods include drip irrigation system (DIS) and nutrient film technique (NFT). Drip irrigation systems involve fertigation systems; in this process, fertilizer and nutrients are dissolved and distributed along with water in drip irrigation (P. Yang et al., 2023). In a hydroponic NFT system, nutrients are supplied to plants by a steady stream of water that forms a 2–3 mm thin film of a nutrient-rich layer. The nutrient supplied to plants is composed of inorganic compounds that produce nutritional ions when dissolved in water (Sharma et al., 2018; Syarifudin et al., 2023).

Various fruits and vegetables have been cultivated using the hydroponic system. For tomatoes, comparisons among NFT, DIS, and the floating raft system revealed superior performance in DIS (Schmautz et al., 2016). Deep water culture produced the highest yield in a lettuce comparative study, while NFT showcased superior water efficiency over conventional soil-based cultivation methods (Majid et al., 2021). Both hydroponic systems produced

significantly higher yield and nutritional content than soil-based cultivation. Deep water culture was also reported to produce tomatoes with higher beta-carotene and lycopene contents compared to DIS and soil-based cultivation (Verdoliva et al., 2021). Collectively, these findings indicate that adopting a hydroponic cultivation system has the potential to enhance crop productivity. Moreover, selecting the appropriate hydroponic system is crucial in achieving optimal results.

In addition to the choice of hydroponic system, the selection of growth media within the system can also substantially impact plant productivity. The choice of media substrate directly influences factors such as root development, nutrient availability, water retention, and aeration, which can collectively affect plant productivity. For example, a coco peat and perlite blend proved most effective when assessing hydroponic substrates for green pepper yield. Additionally, for cucumber cultivation, the pine substrate yielded the highest produce and enhanced the production of essential phytochemicals (Majid et al., 2021; T. Yang et al., 2023).

It is crucial to optimize the system by carefully selecting suitable systems and media substrates tailored to specific plants and environmental conditions to achieve optimal outcomes in hydroponic cultivation. Melon agriculture holds significant economic value globally due to its widespread consumption, market demand, and export potential. While hydroponic systems are widely studied for melon cultivation, research on optimizing the system and growth media remains limited. Additionally, there is a lack of work comparing the DIS and NFT for melon cultivation, especially with varying media in DIS. This study conducts a comprehensive comparison of DIS and NFT for melon cultivation, evaluating outcomes with environmentally friendly, cost-effective, and readily available growth media: rice husk (a rice farming by-product) and compost (from plant and animal waste decomposition) (Gruda, 2019).

## MATERIALS AND METHODS

### Materials

This research used the melon cultivar Madesta (*Cucumis melo* L. cv. Madesta), AB mix nutrients (Hidroponik Surabaya, Indonesia), rice husk and compost. The equipment used for the research includes a greenhouse, drip irrigation and nutrient film technique installation system, a UV-Vis spectrophotometer (Thermo Scientific, USA), and an X-ray fluorescent analyzer (Malvern Panalytical, United Kingdom).

### Experiment Design

Melon was cultivated in a greenhouse using two hydroponic systems: drip irrigation (DIS) and nutrient film technique (NFT). The average temperature inside the greenhouse across the day is relatively stable, between 26–29°C, while humidity is between 67–93%. In DIS, three different growth media were used: rice husk, a mix

of rice husk and compost (1:1), and compost only. The melon seed was first germinated in coco peat media for two weeks. The seedling was transferred to the NFT system and DIS using three different media. In the DIS, AB mix nutrient was supplied to the plant every 5 hr through drip irrigation for 10 min. The AB mix concentration provided to the plants was 750 mg/L in the first and second week of cultivation, 1,000 mg/L in the third week, 1250 mg/L in the fourth week, 1,500 mg/L in the fifth week, and 2,000 mg/L in the sixth week of cultivation. In the NFT system, the AB mix nutrient was supplied continuously to the plants, with the same concentration as used in the DIS. The melon plant was cultivated for a total of 70 days before harvesting. In the fourth week of cultivation, growth parameters were recorded, including leaf number, leaf diameter, plant height, stem diameter, and fruit fresh weight. Following the harvest, physiological parameters were measured, including leaf chlorophyll content, fruit sugar and nitrogen content, and nitrogen level and mineral content in the media before and after cultivation.

## Measurement of Melon Growth Rate

Melon growth parameters were measured every week, from the first week of cultivation in the DIS and NFT system to the fourth week. The growth parameters measured include plant height, leaf number, leaf diameter, and stem diameter.

## Measurement of Leaf Chlorophyll Content

For each treatment, as much as 0.2 g of leaves were collected from three plants. Next, the leaf samples were homogenized, and 10 ml of cold acetone was added. The processed samples were filtered and centrifuged at 4,500 x *g* for 5 min to separate the supernatant. The resulting supernatant was then pipetted into a glass cuvette, and the absorbance was measured at 645 and 662 nm using a UV-Vis spectrophotometer (Thermo Scientific, USA). The chlorophyll content was then calculated using the following equation (Aremu et al., 2012):

$$\text{Chlorophyll } \alpha = (11.24 \times A662) - (2.04 \times A645) \quad (1)$$

$$\text{Chlorophyll } b = (20.13 \times A645) - (4.19 \times A662) \quad (2)$$

$$\text{Total chlorophyll} = (7.05 \times A662) + (18.09 \times A645) \quad (3)$$

## Measurement of Total Nitrogen Content

As much as 0.1 g of dried samples were mixed with 1 g of copper sulfate and 2.5 ml of sulfuric acid. The mixture was then heated for 1 hr using a water bath. The processed samples were then transferred to a distillation flask, and 10 ml of 40% sodium hydroxide (Merck, USA) and 50 ml of distilled water were added; the mixture was then distilled until the volume reached 40 ml. Next, 10 of 0.02 M chloric acid (Merck, USA) was added with 4 drops of red and blue methylene indicators (Merck, USA). The mixture was then titrated by adding 0.02 M sodium hydroxide (Merck, USA) until the solution turned bright green.

### Mineral Content Measurement

As much as 1 g of dried extract was analyzed using an X-ray fluorescent analyzer (Malvern Panalytical, United Kingdom) at 24.7°C and 67% humidity. Two replicates were used to measure the mineral content in each sample.

### Data Analysis

Except for the measurement of mineral content, all measurements were conducted using five to ten independent replicates. The analysis output was presented as mean value ± SD and statistical analysis was performed using two-way analysis of variance (ANOVA) with Duncan's new multiple range test by Statistical Product and Service Solutions (SPSS) software (version 20.0). A probability (*p*-value) of less than 0.05 was considered statistically significant.

## RESULTS

### Melon Cultivation within A Drip Irrigation System, Utilizing A Blend of Rice Husk and Compost, Resulted in Optimal Growth

Overall, the growth measurement data showed that the DIS system has a better effect on melon growth than the NFT system. In the fourth week of the cultivation, melons that were grown using DIS showed higher plant height, number of leaves, leaf diameter, and stem diameter (Figure 1). In the first to fourth week of cultivation, leaf numbers in melons cultivated in NFT are lower than in melons cultivated in DIS using various media (Figure 1A). Similar results were observed for leaf diameter, plant height, and stem diameter in the first three weeks of cultivation (Figures 1B and 1C). On the other hand, in the fourth week of cultivation, stem diameter in the NFT system is not significantly different from that of DIS using compost only and compost mixed with rice husk. However, it is significantly higher than DIS using rice husk only (Figure 1D).

Our data showed that the best media for melon cultivation is DIS, using rice husk mixed with compost (Figure 1), which showed significantly higher leaf diameter and plant height (Figures 1B and 1C) following four weeks of cultivation. However, no significant difference was observed in leaf numbers among different media (Figure 1A).

### The Hydroponic System and Growth Media Influenced Leaf Chlorophyll Content and Fruit Weight

The highest leaf chlorophyll content was observed in melon leaf cultivated using the NFT system, while there is no significant difference in leaf chlorophyll content among different media in DIS (Figure 2A). Although chlorophyll content was highest in plants cultivated using NFT, there is no significant difference in leaf and fruit nitrogen content between plants cultivated in NFT and DIS (Figure 2B). Plants cultivated through NFT, DIS with compost media, and DIS with rice husk mixed with compost displayed comparable fresh fruit weight. However, the fruit weight was notably reduced in plants cultivated using DIS with rice husk media (Figure 2C).

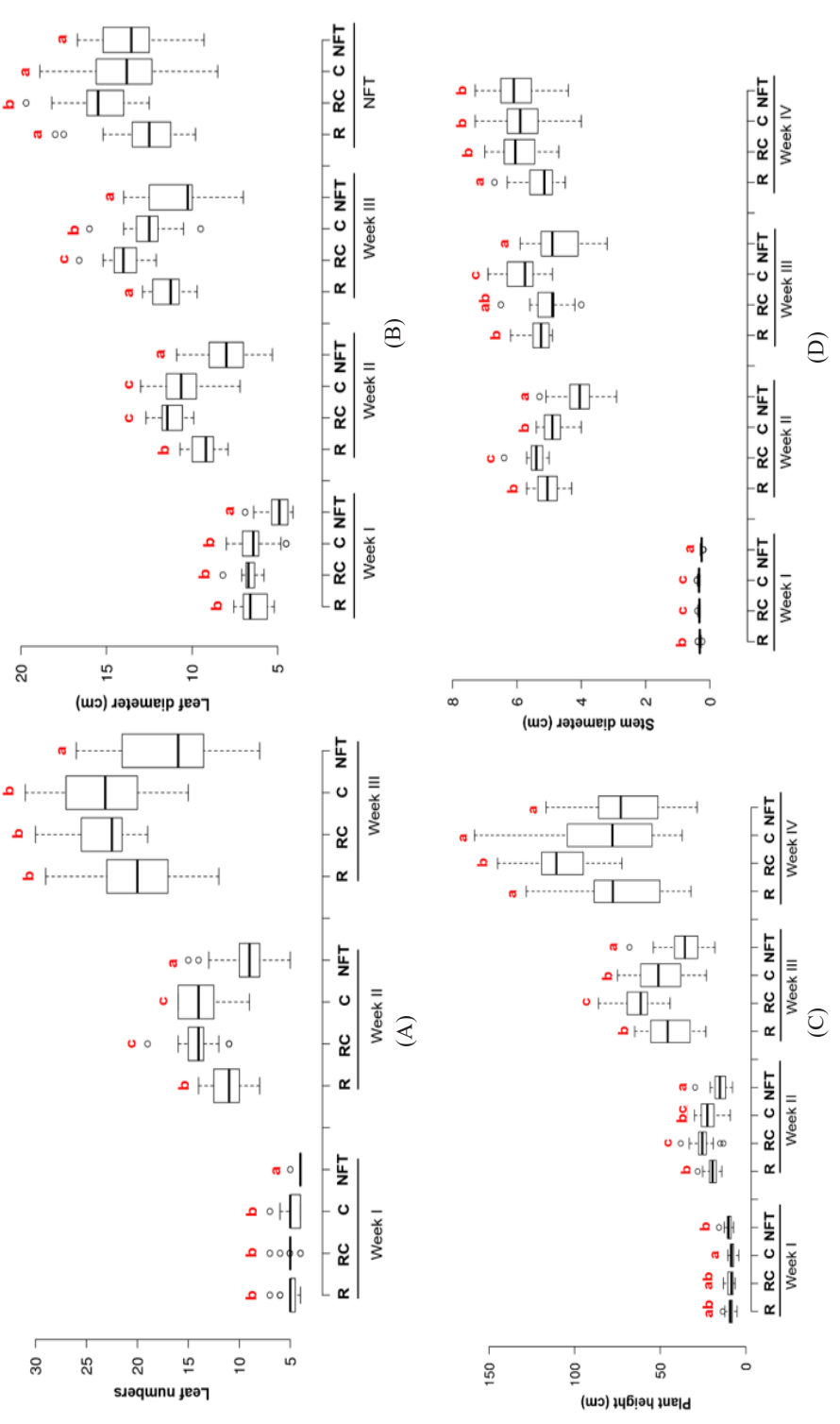



*Figure 1*. The growth parameters of melon following four weeks of cultivation in the nutrient film technique (NFT) system and drip irrigation system (DIS) with different media: (A) Measurement of leaf numbers, (B) Measurement of leaf diameter, (C) Measurement of plant height, and (D) Measurement of stem diameter

*Note*. R = DIS in rice husk, RC = DIS in rice husk + compost, C = DIS in compost only, and NFT = Nutrient film technique. Mean pairs with different letters are significantly different at the 5% probability level according to Duncan's new multiple range test

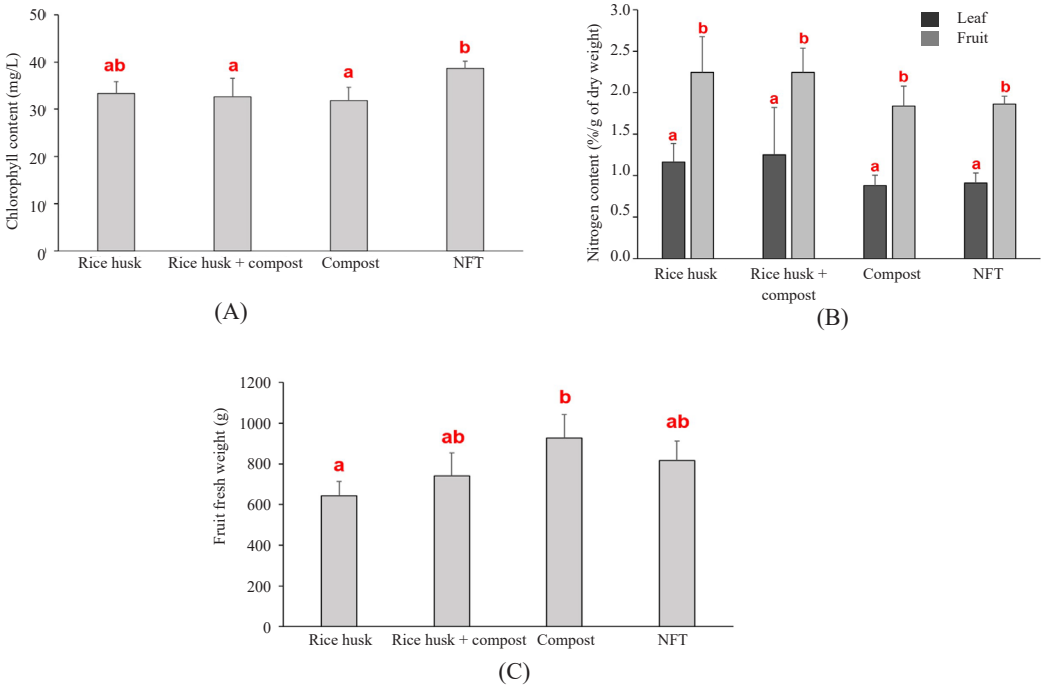



*Figure 2*. The effect of hydroponic system and growth media on (A) chlorophyll content, (B) leaf and fruit nitrogen content, and (C) fruit fresh weight

*Note*. Mean pairs with different letters are significantly different at the 5% probability level, according to Duncan's new multiple range test; NFT = Nutrient film technique

### *Melon Leaves and Fruits Exhibit Varying Mineral Compositions with Different Hydroponic Systems and Media Substrates*

Across all hydroponic systems, aluminum and silicon were only detected in the leaf but not in the fruit (Table 1). The phosphorus concentration in the leaf is higher than in the fruit, and there is no significant difference in phosphorus concentration between plants cultivated in NFT and DIS; it is between 1.8–2.1% in the leaf and 3.8–4.4% in fruit (percent of weight). In all hydroponic systems, the most abundant mineral in melon leaves is calcium (65.9–69.1%), while the most abundant mineral in fruit is potassium (75.9–80%) (Table 1). The highest calcium (69.1%) and potassium (80.1%) concentrations were found in plants cultivated using DIS with rice husks mixed with compost media. These findings indicate that selecting hydroponic systems and media substrates can influence the mineral content in melon leaves and fruits.

### Initial and Post-harvest Nitrogen and Mineral Content in the Growth Media

The initial nitrogen content in the media was similar between rice husk and rice husk mixed with compost, but it was significantly lower in media with compost only (Figure 3). Following cultivation and harvest, the

Table 1
*Mineral content (% of dry weight) in the leaf and fruit of melon cultivated using NFT system and DIS with different media*

| Mineral | Leaf (% of dry weight) | | | | Fruit (% of dry weight) | | | |
|---|---|---|---|---|---|---|---|---|
| | R | RC | C | NFT | R | RC | C | NFT |
| Al | 5.5 ± 0.7 | 5.5 ± 0.7 | 5.5 ± 0.7 | 5.5 ± 0.7 | 0 | 0 | 0 | 0 |
| Si | 4.5 ± 0.2 | 3.5 ± 0.6 | 4.2 ± 0.7 | 2.7 ± 0.0 | 0 | 0 | 0 | 0 |
| P | 1.9 ± 0.1 | 1.8 ± 0.6 | 2.1 ± 0.5 | 2.1 ± 0.0 | 3.9 ± 0.2 | 3.8 ± 0.0 | 4.4 ± 0.0 | 4.2 ± 0.4 |
| S | 3.7 ± 0.1 | 3.8 ± 0.2 | 3.8 ± 0.9 | 3.4 ± 0.4 | 1.6 ± 0.0 | 1.7 ± 0.1 | 1.7 ± 0.6 | 1.6 ± 0.2 |
| K | 15.6 ± 0.8 | 14.7 ± 0.2 | 17 ± 0.1 | 18.8 ± 0.2 | 75.9 ± 0.1 | 80.1 ± 0.4 | 77.3 ± 0.1 | 79.9 ± 0.2 |
| Ca | 67.3 ± 0.2 | 69.1 ± 0.2 | 65.9 ± 0.3 | 66.3 ± 0.8 | 16.4 ± 0.1 | 12.4 ± 0.2 | 14.3 ± 0.1 | 12.1 ± 0.1 |
| Mn | 0.1 ± 0.1 | 0.3 ± 0.1 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.1 ± 0.0 |
| Fe | 0.7 ± 0.0 | 0.6 ± 0.0 | 0.7 ± 0.0 | 0.7 ± 0.0 | 0.5 ± 0.0 | 0.4 ± 0.0 | 0.5 ± 0.0 | 0.5 ± 0.0 |
| Cu | 0.2 ± 0.0 | 0.3 ± 0.0 | 0.3 ± 0.0 | 0.3 ± 0.0 | 0.6 ± 0.0 | 0.6 ± 0.0 | 0.7 ± 0.0 | 0.7 ± 0.0 |
| Zn | 0 | 0 | 0 | 0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 |
| Br | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rb | 0 | 0 | 0 | 0 | 0.2 ± 0 | 0.2 ± 0 | 0 | 0 |
| Sr | 0.3 ± 0.0 | 0.3 ± 0.0 | 0.3 ± 0.0 | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0 | 0.1 ± 0.0 | 0.1 ± 0.0 |
| Re | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.4 ± 0.0 | 0.5 ± 0 | 0.5 ± 0.0 | 4.3 ± 0.1 |

*Note*. R = DIS in rice husk; RC = DIS in rice husk + compost; C = DIS in compost only; NFT = Nutrient film technique; DIS = Drip irrigation system

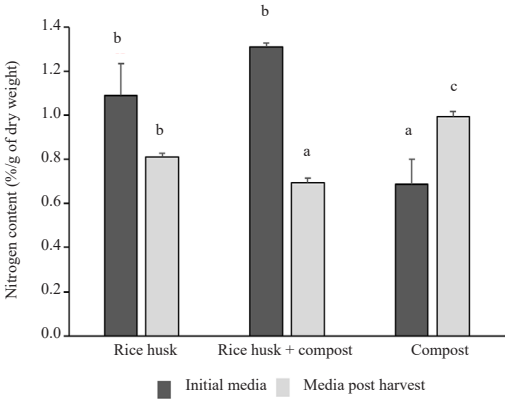



*Figure 3*. Initial and post-harvest nitrogen content in the growth media

*Note*. Mean pairs with different letters are significantly different at the 5% probability level, according to Duncan's new multiple range test

nitrogen content in media with compost is the highest, followed by rice husk and rice husk mixed with compost (Figure 3).

Silica was observed as the most abundant media in the initial media. Silica concentration is highest in rice husk in initial and post-harvest media. The concentration of silica, phosphorous, sulfur, and potassium is higher in post-harvest media compared to the initial media. On the other hand, the level of manganese is slightly decreasing in post-harvest media, while iron content is reduced significantly. The concentration of other minerals is very low across all media used, both in initial and post-harvest media (Table 2).

## DISCUSSION

During the initial stages of melon growth, plants in the DIS exhibit better growth compared to the NFT system, evident in higher leaf numbers, leaf diameter, plant height, and stem diameter. These results indicate that solid growth media is required to support melon growth, especially to facilitate root development. The plants are growing slower in the NFT system because they need to adapt to the new environment since the plants are grown on liquid media, while melons naturally grow on soil. It aligns with prior studies that have indicated that media for hydroponic systems should have good water retention capacity, be rich in nutrients, have good aeration, and have a high ion exchange capacity.

Across the three media used in DIS, the best growth was observed in melon cultivated using rice husk mixed with compost. When grown in this media, the plants showed the highest leaf diameter, plant height, and stem diameter because compost mixed with rice husk has a high water retention capacity, good aeration and drainage, and sufficient nutrition. Compost is an organic material produced through thermophilic and aerobic decomposition, rich with nutrients required by plants and an environmentally friendly fertilizer (Raviv, 2017). Media consisting of rice husks might have good aeration and drainage but have relatively low water retention capacity. Therefore, the supplied nutrient-rich water is not effectively retained in the media and absorbed by the root, thus limiting the melon growth. Media consisted only of compost, limiting the growth of melon because it has low aeration and drainage capacity. In previous work, it was reported that the best media for Inpari rice cultivation in a hydroponic system is rice husk charcoal and

Table 2
*Mineral content (% of dry weight) in DIS initial and post-harvest media*

| Mineral | R | RC | C | R | RC | C |
|---|---|---|---|---|---|---|
| | | Initial media | | | Post-harvest media | |
| Al | 0.9 ± 0.1 | 5.2 ± 0.4 | 6.9 ± 0.3 | 0.8 ± 0.2 | 2.8 ± 0.3 | 4.9 ± 0.1 |
| Si | 57.3 ± 0.1 | 39.9 ± 0.1 | 35.7 ± 0.1 | 56.2 ± 0.3 | 55.6 ± 0.1 | 46.6 ± 0.1 |
| P | 3.1 ± 0.1 | 2.3 ± 0.1 | 2.1 ± 0.1 | 3.8 ± 0.2 | 2.7 ± 0.0 | 2.3 ± 0.0 |
| S | 1.6 ± 0.0 | 0.8 ± 0.0 | 0.6 ± 0.0 | 2.8 ± 0.0 | 1.4 ± 0.1 | 0.9 ± 0.0 |
| K | 11.4 ± 0.1 | 5 ± 0.0 | 4.1 ± 0.0 | 11.7 ± 01 | 5.4 ± 0.0 | 4.5 ± 0.0 |
| Ca | 13.1 ± 0.1 | 12.6 ± 0.1 | 13 ± 0.0 | 15.7 ± 0.1 | 10.2 ± 0.1 | 11.1 ± 0.1 |
| Ti | 0.4 ± 0.0 | 1.8 ± 0.0 | 2.1 ± 0.0 | 0.2 ± 0.0 | 1.2 ± 0.0 | 1.7 ± 0.0 |
| V | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0 | 0 | 0.1 ± 0.0 |
| Mn | 2.9 ± 0.0 | 1.2 ± 0.0 | 0.9 ± 0.0 | 2.3 ± 0.0 | 0.9 ± 0.0 | 0.9 ± 0.0 |
| Fe | 7.7 ± 0.1 | 29.8 ± 0.3 | 33.1 ± 0.1 | 5.1 ± 0.0 | 18.5 ± 0.1 | 25.9 ± 0.1 |
| Cu | 0.6 ± 0.0 | 0.4 ± 0.0 | 0.3 ± 0.0 | 0.5 ± 0.0 | 0.3 ± 0.0 | 0.3 ± 0.0 |
| Zn | 0.9 ± 0.0 | 0.2 ± 0.0 | 0.2± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 |
| Sr | 0.1 ± 0.0 | 0.4 ± 0.0 | 0.4± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.3 ± 0.0 |
| Zr | 0 | 0.1 ± 0.0 | 0.1± 0.0 | 0 | 0.1 ± 0.0 | 0.1± 0.0 |
| Ba | 0.2 ± 0.0 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.3 ± 0.0 |
| Re | 0.4 ± 0.0 | 0.1 ± 0 | 0.1 ± 0.0 | 0.3 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 |

*Note*. R = DIS in rice husk; RC = DIS in rice husk + compost; C = DIS in compost only; NFT = Nutrient film technique; DIS = Drip irrigation system

cocopeat (Hidayat et al., 2021), while the media consisting of coconut husk is better for tomato compared to peat vermiculite and rock wool (Xiong et al., 2017). Collectively, these findings imply that each plant species required specific media for their optimal growth.

Although melon growth is slower in the NFT system, the chlorophyll content of NFT plants is higher than that of DIS. In the NFT system, AB mix nutrition is supplied continuously. Thus, nutrient availability for chlorophyll production is higher. In accordance with this, it is also reported that in lettuce, cultivation using NFT also resulted in higher leaf chlorophyll content compared to lettuce grown in soil and in aggregate media hydroponic system (Thomas et al., 2021). Total nitrogen measurement showed that the highest nitrogen level is found in med with rice husk mixed with compost (1.3%), while the lowest is found in compost media (0.7%). The high nitrogen content in mixed media is due to the high protein content in rice husk. Kuan et al. (2012) reported that dried rice husk contains 71.6% carbohydrate, 5.5% protein, 15.4% ash, and 0.9% fat. The application of rice husk biochar was also reported to improve nitrogen availability in soil (Oladele et al., 2019; Selvarajh et al., 2021). Low nitrogen content in compost media is probably due to incomplete decomposition of plant materials and animal waste in the compost. Therefore, the complex organic matter is not yet decomposed to simple nitrogen compounds.

Our data showed that the post-harvest media contained less nitrogen than the initial media, except for compost-only media, which showed higher nitrogen levels in post-harvest media. In rice husk media, we observed a 28% reduction in nitrogen content, while in rice husk mixed with compost, 38% nitrogen reduction was observed. On the other hand, a 30% increase in nitrogen content was detected in the compost post-harvest media (Figure 2C). The increasing nitrogen content in compost-only post-harvest media is probably due to low aeration and drainage; the supplied nitrogen-rich AB mix solution is trapped in the media and not effectively absorbed into the root. Our data indicates that AB mix solution can provide sufficient nitrogen for melon hydroponic cultivation, as shown by the consistently high nitrogen content in the fruit and leaf of melon cultivated using the NFT and DIS system. Although nitrogen content remains similar across all treatments, the significant differences in fruit weight were observed. The highest fruit weight was recorded in plants cultivated using DIS with compost-only media, while the lowest was observed in DIS with rice husk media. The volume and concentration of AB mix nutrients provided to the plants significantly affect the fruit's weight. Further optimization is required to evaluate the best volume, concentration, and period for the AB mix application.

The mineral content in the media is relatively stable, and there are no significant mineral content differences between initial and post-harvest media. This result indicates that melon is acquiring minerals mainly from the supplemented AB mix and not from the

media. Some minerals are detected at higher concentrations in fruits compared to leaves; for example, phosphorus concentration in leaves (1.8-2.1%) is significantly lower than in fruits (3.8-4.4%). In general, phosphorus content is higher in fruits than in leaves, as observed in soybeans, almonds, and wheat (Siregar, 2020). Potassium concentration is also higher in melon fruits (76–80%) compared to leaf (14.70–18.85%). Potassium is required in protein synthesis (Koch & Mengel, 1974); the potassium level in leaves is lower than in fruits because proteins are synthesized at much higher levels since the development of seeds and fruits requires the activity of many different enzymes.

The micronutrient that is detected at different concentrations between leaf (0.59–0.73%) and fruits (0.10–0.19%) is iron. Although iron is not a direct part of chlorophyll structure, it is required to convert magnesium protoporphyrin to protochlorophyllide in chlorophyll. Therefore, iron is a pivotal component of chlorophyll synthesis (George et al., 1995). Due to iron's function in chlorophyll synthesis, iron is found in a much higher concentration in the leaf than in fruit.

## CONCLUSION

This study identifies the DIS with a substrate of rice husk mixed with compost (1:1) as the optimal hydroponic system for enhancing melon growth and yield. While melon growth is relatively slower in the NFT system, the chlorophyll content in NFT plant leaves is higher. There are no significant differences in nitrogen content between NFT and DIS systems. However, potassium and calcium levels are higher in melons cultivated in DIS with a mix of rice husk and compost. A distinct variance in mineral content is observed between melon leaves and fruits, with Fe and calcium being predominant in leaves and potassium dominating in fruits.

## ACKNOWLEDGEMENTS

This research is funded by Riset Kolaborasi Indonesia-WCU (World Class University Tahun Anggaran 2023), number 967/UN3. LPPM/PT.01.03/2023

## REFERENCES

Aremu, A. O., Bairu, M. W., Szüčová, L., Doležal, K., Finnie, J. F., & Staden, J. V. (2012). Assessment of the role of *meta*-topolins on *in vitro* produced phenolics and acclimatization competence of micropropagated 'Williams' banana. *Acta Physiologiae Plantarum*, *34*, 2265–2273. https://doi.org/10.1007/s11738-012-1027-6

George, E., Marschner, H., & Jakobsen, I. (1995). Role of arbuscular mycorrhizal fungi in uptake of phosphorusus and nitrogen from soil. *Critical Reviews in Biotechnology*, *15*(3–4), 257–270. https://doi.org/10.3109/07388559509147412

Gruda, N. S. (2019). Increasing sustainability of growing media constituents and stand-alone substrates in soilless culture systems. *Agronomy*, *9*(6), 298. https://doi.org/10.3390/agronomy9060298

Hidayat, C., Frasetya, B., & Lestari, F. A. (2021). Effects of growth media compositions for plant growth of Inpari 13 rice (*Oryza sativa* L.) on substrate hydroponic system. In *IOP Conference Series: Materials Science and Engineering* (Vol. 1098, No. 5, p. 052007). IOP Publishing. https://doi.org/10.1088/1757-899X/1098/5/052007

Koch, K., & Mengel, K. (1974). The influence of the level of potassium supply to young tobacco plants (*Nicotiana tabacum* L.) on short-term uptake and utilisation of nitrate nitrogen ($^{15}$ N). *Journal of the Science of Food and Agriculture*, *25*(5), 465–471. https://doi.org/10.1002/jsfa.2740250504

Kuan, C.-Y., Yuen, K.-H., & Liong, M.-T. (2012). Physical, chemical and physicochemical characterization of rice husk. *British Food Journal*, *114*(6), 853–867. https://doi.org/10.1108/00070701211234372

Majid, M., Khan, J. N., Shah, Q. M. A., Masoodi, K. Z., Afroza, B., & Parvaze, S. (2021). Evaluation of hydroponic systems for the cultivation of lettuce (*Lactuca sativa* L., var. Longifolia) and comparison with protected soil-based cultivation. *Agricultural Water Management*, *245*, 106572. https://doi.org/10.1016/j.agwat.2020.106572

Oladele, S. O., Adeyemo, A. J., & Awodun, M. A. (2019). Influence of rice husk biochar and inorganic fertilizer on soil nutrients availability and rain-fed rice yield in two contrasting soils. *Geoderma*, *336*, 1-11. https://doi.org/10.1016/j.geoderma.2018.08.025

Raviv, M. (2017). Can compost improve sustainability of plant production in growing media? *Acta Horticulturae*, *1168*, 119–134. https://doi.org/10.17660/ActaHortic.2017.1168.17

Schmautz, Z., Loeu, F., Liebisch, F., Graber, A., Mathis, A., Bulc, T. G., & Junge, R. (2016). Tomato productivity and quality in aquaponics: Comparison of three hydroponic methods. *Water*, *8*(11), 533. https://doi.org/10.3390/w8110533

Selvarajh, G., Ch'ng, H. Y., Md Zain, N., Sannasi, P., & Mohammad Azmin, S. N. H. (2021). Improving soil nitrogen availability and rice growth performance on a tropical acid soil via mixture of rice husk and rice straw biochars. *Applied Science*, *11(1)*, 108. https://doi.org/10.3390/app11010108

Sharma, N., Acharya, S., Kumar, K., Singh, N., & Chaurasia, O. P. (2018). Hydroponics as an advanced technique for vegetable production: An overview. *Journal of Soil and Water Conservation*, *17*(4), 364–371. https://doi.org/10.5958/2455-7145.2018.00056.5

Siregar, R. M. (2020). Analysis of fosfor hara elements in coconut leaves oil by spectrofotometry. *Indonesian Journal of Chemical Science and Technology*, *3*(1), 35-37. https://doi.org/10.24114/ijcst.v3i1.18547

Syarifudin, A. A., Prayogo., Suciyono., Kenconojat, H., Santanumurti, M. B., Lamadi, A., & Jati, C. W. (2023). Performance of climbing perch (*Anabas testudineus*) and bok choy (*Brassica chinensis*) in aquaponics systems using nutrient film technique in Indonesian small-scale livestock. *Pertanika Journal of Tropical Agricultural Science*, *46*(4), 1375-1390. https://doi.org/10.47836/pjtas.46.4.19

Thomas, T., Biradar, M. S., Chimmad, V. P., & Janagoudar, B. S. (2021). Growth and physiology of lettuce (*Lactuca sativa* L.) cultivars under different growing systems. *Plant Physiology Report*, *26*, 526–534. https://doi.org/10.1007/s40502-021-00591-3

Velazquez-Gonzalez., R. S., Garcia-Garcia., A. L., Ventura-Zapata., E., Barceinas-Sanchez, J. D. O., & Sosa-Savedra, J. C. (2022). A review on hydroponics and the technologies associated for medium-and small-scale operations. *Agriculture*, *12*(5), 646. https://doi.org/10.3390/agriculture12050646

Verdoliva, S. G., Gwyn-Jones, D., Detheridge, A., & Robson, P. (2021). Controlled comparisons between soil and hydroponic systems reveal increased water use efficiency and higher lycopene and β-carotene contents in hydroponically grown tomatoes. *Scientia Horticulturae*, *279*, 109896. https://doi.org/10.1016/j.scienta.2021.109896

Xiong, J., Tian, Y., Wang, J., Liu, W., & Chen, Q. (2017). Comparison of coconut coir, rockwool, and peat cultivations for tomato production: Nutrient balance, plant growth and fruit quality. *Frontiers in Plant Science*, *8*, 1327. https://doi.org/10.3389/fpls.2017.01327

Yang, P., Wu, L., Cheng, M., Fan, J., Li, S., Wang, H., & Qian, L. (2023). Review on drip irrigation: Impact on crop yield, quality, and water productivity in China. *Water*, *15*(9), 1733. https://doi.org/10.3390/w15091733

Yang, T., Altland, J. E., & Samarakoon, U. C. (2023). Evaluation of substrates for cucumber production in the Dutch bucket hydroponic system. *Scientia Horticulturae*, *308*, 111578. https://doi.org/10.1016/j.scienta.2022.111578

Pertanika J. Trop. Agri. Sci. 47 (4): 1205 - 1219 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Effects of Harvest Time and *Acacia crassicarpa* Age on the Physicochemical Characteristics of *Apis mellifera* L. Honey in Tropical Indonesian Forests

**Eni Suhesti[1,2], Lili Zalizar[1], Joko Triwanto[1], Ervayenri Ervayenri[2] and Indra Purnama[3,4]***

*[1]Department of Postgraduate, University of Muhammadiyah Malang, Malang 65144, East Java, Indonesia*
*[2]Department of Forestry, Faculty of Forestry and Sciences, Universitas Lancang Kuning, Pekanbaru 28261, Riau, Indonesia*
*[3]Graduate School of Agricultural Sciences, Universitas Lancang Kuning, Pekanbaru 28261, Riau, Indonesia*
*[4]Center for Sustainable Tropical Agricultural Research, Universitas Lancang Kuning, Pekanbaru 28261, Riau, Indonesia*

## ABSTRACT

The comprehensive understanding of the physicochemical profile of monofloral honey derived from *Acacia crassicarpa*, specifically in the Indonesian tropical forest ecosystem, has not been fully explored. The physicochemical characteristics of honey significantly influence its quality and consumer acceptance. Harvest time and the age of *Acacia* plants, which are suspected to affect honey's physicochemical properties, are this study's focal points. Our objective is to analyze the impact of harvest time and *Acacia* age on the physicochemical characteristics of honey. Using a complete randomized block design, treatments were administered at 14, 21, and 30 days of harvest within three *Acacia* age groups: 3, 8, and 18 months. The honey composition was assessed following the Indonesian National Standard 8664:2018 procedure. The statistical analysis determined the optimal harvest period for honey by assessing its physicochemical properties and comparing them to the Indonesian National Standard 8664:2018 procedure (SNI 8664:2018 standards). One-way analysis of variance evaluated the effects of harvest time and plant age on composition, followed by a least significant difference tests to identify significant differences between harvest times. Results indicate a significant influence of harvest time and Acacia age on all honey composition variables, including diastase

ARTICLE INFO

*Article history:*
Received: 19 February 2024
Accepted: 18 April 2024
Published: 25 October 2024

DOI: https://doi.org/10.47836/pjtas.47.4.10

E-mail addresses:
suhestieni@unilak.ac.id (Eni Suhesti)
lily@umm.ac.id (Lili Zalizar)
joko.fpumm@gmail.com (Joko Triwanto)
erva@unilak.ac.id (Ervayenri)
indra.purnama@unilak.ac.id (Indra Purnama)
*Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

enzyme activity, hydroxymethylfurfural content, moisture level, sugar content, and acidity ($P<0.01$). Our findings suggest optimal honey harvest at 30 days, aligning with the 8th and 18th months of *A. crassicarpa*. Most variables met SNI 8664:2018 standards, except acidity levels. Further investigation is needed to discern the causes of acidity in *Apis mellifera* honey from Indonesian peat swamp forests.

*Keywords*: *Acacia crassicarpa*, *Apis mellifera*, monofloral honey, peat swamp forest, tropical forest honey

## INTRODUCTION

Honey is a sweet liquid with a complex composition comprising carbohydrates and other compounds (De-Melo et al., 2018; Hailu & Belay, 2020). The composition of honey is influenced by the type of plant forage, bee species, environmental conditions, geographical location, handling procedures, and storage methods (Alvarez-Suarez et al., 2018; Baroni et al., 2015; Viteri et al., 2021). Based on the diversity of bee forage plant types, honey is classified into monofloral honey and polyfloral honey (Hailu & Belay, 2020). Monofloral honey is produced by honeybees with forage from a single plant species. Generally, monofloral honey is preferred by consumers over multifloral honey due to reasons related to taste, aroma, and health attributes (Ghramh et al., 2023; Taha et al., 2021).

Studies on the physicochemical properties of monofloral honey from various forage plant species have been extensively conducted previously in countries such as Romania, Brazil, and Hungary (Czipa et al., 2019; do Nascimento et al., 2018; Oroian & Sorina, 2017). Information regarding the physicochemical characteristics of honey is crucial as it significantly influences honey's nutritional quality, taste, texture, and health value (Oroian & Sorina, 2017; Siddiqui et al., 2017). *Acacia crassicarpa* honey in Riau has a rich history, with beekeeping practices becoming popular in recent years, particularly during the COVID-19 pandemic due to increased demand (Purwanto et al., 2024). Meanwhile, the production of *A. crassicarpa* honey is declining due to reduced natural forest area (Pribadi & Wiratmoko, 2023).

In Indonesia, monofloral honey derived from the extrafloral nectar of *A. crassicarpa* is predominantly produced by local beekeepers utilizing *A. mellifera* within acacia plantation forest areas, including Siak Regency, Riau Province, Indonesia. This region is characterized by peat swamp forests with distinctive acidic soil properties. Local honey is widely marketed within Riau Province and other provinces in Indonesia. Indonesian beekeepers face challenges in consistently producing honey that adheres to the quality benchmarks established by the Indonesian National Standard (Standard Nasional Indonesia, SNI) 8664-2018. This issue is particularly concerning for industrial consumers and international buyers, who require honey that meets specific quality criteria. Therefore, a comprehensive investigation into the factors causing the honey quality from these

beekeeping practices to fall short of the standards is imperative.

The honey harvesting practices among *A. mellifera* beekeepers in Siak Regency, Indonesia, exhibited variability attributed to several factors, including meteorological conditions, workforce availability, and fluctuations in consumer demand. Based on observation, the interval between harvests typically ranged from 14 to 30 days. Additionally, *A. crassicarpa* plantations aged between 3 and 18 months were predominantly selected as apiary locations. Hence, it can be hypothesized that these combinations influence the honey's physicochemical properties. According to research by Lewkowski et al. (2019), the maturity level of honey within the hive and the age of the plants serving as the primary forage source for bees are believed to influence honey composition. The timing of honey harvest demonstrably influences its maturity (Wu et al., 2022). Wu et al. (2022) employed a research design to investigate the impact of harvest time on the physicochemical properties of honey produced by two stingless bee species, *Heterotrigona itama* and *Tetrigona binghami*, and observed significant variations. Observations and interviews with local beekeepers suggest that different ages of *A. crassicarpa* tend to yield varying amounts of nectar and may exhibit different characteristics. Nonetheless, a comprehensive understanding of how harvest timing and the age of *A. crassicarpa* influence the composition of *A. mellifera* honey in tropical forests remains elusive. This knowledge gap necessitates further rigorous investigation.

This study addresses this knowledge gap by specifically investigating the influence of harvest time and the age of *A. crassicarpa* on the physicochemical characteristics of *A. mellifera* honey. *Acacia crassicarpa*, chosen for its ecological significance and potential impact on honey composition, is a prominent nectar source in the tropical forests of Indonesia. Understanding the intricate relationship between harvesting period, plant maturity, and honey composition is crucial for optimizing apicultural practices, particularly within peat swamp forests, and ensuring the production of high-quality honey. Explaining the complicated relationship between harvesting period, plant maturity, and honey composition is principal to optimizing apicultural practices, especially within peat swamp forests, and guaranteeing the production of honey with superior quality. The objectives of this study are: 1) to analyze the influence of harvest timing, *A. crassicarpa* age, and the interaction between harvest timing and *A. crassicarpa* age on physicochemical characteristics of honey, and 2) to determine the optimal harvest timing and plant age for *A. mellifera* honey produced by communities in Siak Regency, Riau.

## MATERIALS AND METHODS

### Experimental Design

The experimental design employed a factorial design. The honey samples were categorized based on three distinct harvest times (14, 21, and 30 days) and

further stratified into three age groups of *A. crassicarpa* (3, 8, and 18 months). All treatments were replicated three times, resulting in 27 experimental units. This study employed a research design that specifically accounted for the variation introduced by harvest timing, plant age, and interaction between harvest time and plant age. This comprehensive approach allowed for a robust assessment of how these factors influence honey physiochemical composition.


*Figure 1*. Overview of the study with image descriptions: (A) *Acacia crassicarpa* tree at 3 months old; (B) Bees are collecting extrafloral nectar of *A. crassicarpa*; (C) Honeycomb being lifted from the beehive box; and (D) A honey extractor functions to mechanically separate honey from its frames, facilitating the collection of fresh honey

## Honey Samples Preparation

The honey samples used in this study originated from bee yards located within *A. crassicarpa* plantations managed by Arara Abadi Co., Ltd., Siak Regency, Riau, Indonesia (0°48'32.21057"N 101°36'22.85467"E). The honey sampling was conducted in May 2023, during the dry season, with an average temperature of 32.6°C prevailing throughout the research period. Honey samples were collected from bee yards placed within three age groups (3, 8, and 18 months) of *A. crassicarpa* plantations. Each age group of plantations encompassed a minimum area ranging from 100 to 200 ha. Therefore, it was assumed that *A. mellifera* colonies placed within one age group of *A. crassicarpa* plantations would not fly to other age groups of plantations. This assumption is based on

the factors that influence bee flight distance, including nectar's adequacy and quality (Tong et al., 2019). *Acacia crassicarpa* plants produce a considerable amount of nectar, averaging 42,774 ml/ha per day for 12-month-old plants and 73,766 ml/ha per day for 50-month-old plants (Pribadi & Purnomo, 2013).

Harvesting took place at 8 a.m. to ensure consistency and account for diurnal variations following the previous study (Pasias et al., 2018). The process of fresh honey retrieval involved meticulous combing of the hives and extraction of honeycombs using a mechanical honey extractor for efficient honey extraction. The freshly extracted honey, collected through this process, was promptly transferred to sterile glass vessels. These vessels were sealed and shielded from light to prevent alterations in honey composition during transportation to the laboratory. Honey samples were stored at room temperature in dark conditions, as suggested by Radtke and Lichtenberg-Kraag (2018). This controlled environment guaranteed the stability of the honey samples until the initiation of detailed physicochemical analysis. Figure 1 illustrates an overview of the research in this study.

**Physicochemical Analysis**

All honey samples were handled with precision, and the utmost care was taken to maintain their purity. Notably, analytical-grade chemicals were exclusively used throughout the entire process to ensure accuracy and reliability in the analysis. The physicochemical analysis procedures, diastase activity, determination of hydroxymethylfurfural (HMF), moisture content, sucrose content, reducing sugar content, and acidity for honey composition followed established protocols as outlined in Berhe et al. (2018) and Suhesti, Zalizar, et al. (2023). Determining diastase enzyme activity and HMF content utilized spectrophotometry methods, while refractometry methods measured moisture levels. The analysis of reducing sugar (glucose) and sucrose content employed the Luff-Schoorl method, and acidity was assessed using the neutralization method (Suhesti, et al., 2023).

**Data Analysis**

The influence of harvest time, the age of *A. crassicarpa* plants, and the interaction between these two factors (age and harvest time) on honey physicochemical properties was analyzed using the one-way analysis of variance (ANOVA) and least significant difference (LSD) post-hoc test in SPSS (version 25). This analysis determines the optimal harvest time and plant age for each aspect of honey composition. Additionally, one sample *t*-test was employed to compare the mean values of honey composition variables with the quality standards specified by SNI 8664-2018. Quantitative descriptive analysis was conducted to illustrate the mean values, standard deviations, and comparisons with the quality standards.

## RESULTS AND DISCUSSION

### Diastase Activity and HMF Determination

Enzyme activity, specifically diastase and HMF levels, indicates honey freshness (Al-Ghamdi et al., 2019; Bell & Grainger, 2023). The diastase enzyme activity value is crucial in assessing honey quality and is closely associated with its nutritional quality and freshness (Can et al., 2015; Erban et al., 2021). Elevated levels of HMF in honey may indicate adulteration, such as adding inverted sugar syrup (Pasias et al., 2018; Yücel & Sultanoğlu, 2013).

The one-way ANOVA revealed a significant influence ($P<0.01$) of harvest time, the age of *A. crassicarpa* plants, and the interaction between harvest time and age of plants on diastase enzyme activity and HMF level (Table 1). According to the LSD test, diastase enzyme activity exhibited significant differences among harvest times of 14, 21, and 30 days across all plant ages ($P<0.01$) (Table 1). Conversely, harvest time had a significant effect on the HMF values of *A. crassicarpa* at 3 and 8 months of age ($P<0.01$). However, this effect was not significant at 18 months of age ($P>0.05$) (Table 1).

The analysis of diastase enzyme activity in honey revealed an interaction between harvest time and the age of *A. crassicarpa*, with the most optimal combination being 30 days of harvest time and 3 months of plant age, yielding a result of $11.21 \pm 0.02$ diastase number (DN). The one sample *t*-test results indicated a significant difference between this value and the standard specified in SNI 8664-2018 ($P<0.01$), which requires a minimum of 3 DN, meeting export requirements set at above 8 DN (Bell & Grainger, 2023). Furthermore, this value aligns with the diastase enzyme activity range reported by Sajid et al. (2019) in their study on fresh honey from various regions in Pakistan, ranging from 10.70 to 23.00 DN.

The tendency for higher diastase enzyme activity in honey harvested over a longer period is likely due to the extended honey ripening process, leading to increased secretion of enzymes from the bee's stomach. It aligns with the statement by Eyer et al. (2016) that honey bees release α-amylase enzymes during nectar collection and ripening into honey. Diastase enzyme activity is a combination of α-amylase and β-amylase activities secreted from the bee's saliva, playing a crucial role in honey production by aiding in the conversion of starch into maltose (Chua & Adnan, 2014; Sajid et al., 2019).

The one-way ANOVA revealed a significant influence ($P<0.01$) of harvest time, the age of *A. crassicarpa* plants, and the interaction between harvest time and age of plants on HMF value (Table 1). Based on the results of the one-sample *t*-test, all honey samples from every harvest time and plant age group exhibited significant differences compared to the SNI 8664-2018 standard ($P<0.01$), meeting the established criteria. Interestingly, honey derived from *A. mellifera* foraging for three months *A. crassicarpa* exhibited an increase of HMF value when harvest time was extended. It aligns with the findings of research

Table 1
*Physicochemical data of fresh honey samples from various acacia plant ages and harvest times*

| Parameters | Honey samples | | | | | |
|---|---|---|---|---|---|---|
| | 3 months | | | 8 months | | |
| | 14 days (n = 3) | 21 days (n = 3) | 30 days (n = 3) | 14 days (n = 3) | 21 days (n = 3) | 30 days (n = 3) |
| Diastase activity (DN) | 1.95 ± 0.03a** | 2.54 ± 0.04b** | 11.21 ± 0.02c | 2.55 ± 0.05b** | 6.55 ± 0.10c | 1.52 ± 0.02a** |
| HMF-content (mg/kg) | 0.00 ± 0.00a | 0.00 ± 0.00a | 1.87 ± 0.09b | 0.00 ± 0.00b | 0.00 ± 0.00b | 0.00 ± 0.00a |
| Moisture (%) | 26.40 ± 0.40c** | 23.87 ± 0.30b** | 20.25 ± 0.20a | 23.87 ± 0.35b** | 23.20 ± 0.10b** | 22.20 ± 0.10a** |
| Sucrose content (w/w) | 12.05 ± 0.50a** | 7.76 ± 0.01b** | 19.55 ± 0.40a** | 14.19 ± 0.40b** | 14.91 ± 0.01c** | 1.50 ± 0.30a |
| Glucose content (w/w) | 27.21 ± 0.20a** | 27.80 ± 0.10a** | 61.15 ± 0.05b** | 58.13 ± 0.20b** | 52.10 ± 0.10a** | 66.56 ± 0.04c |
| Acidity (NaOH/kg) | 92.15 ± 0.15** | 70.01 ± 0.07** | 59.41 ± 0.10** | 114.76 ± 0.02** | 131.97 ± 0.05** | 113.05 ± 0.05** |

| Parameters | honey sample | | |
|---|---|---|---|
| | 18 months | | |
| | 14 days (n = 3) | 21 days (n = 3) | 30 days (n = 3) |
| Diastase activity (DN) | 3.91 ± 0.01c | 2.80 ± 0.05b** | 1.23 ± 0.05a** |
| HMF-content (mg/kg) | 0.00 ± 0.00a | 0.00 ± 0.00a | 0.000.00a |
| Moisture (%) | 22.60 ± 0.25b** | 21.80 ± 0.2b | 21.00 ± 0.10a |
| Sucrose content (w/w) | 19.55 ± 0.40c** | 14.08 ± 0.01b** | 5.00 ± 0.30a |
| Glucose content (w/w) | 53.28 ± 0.30a** | 52.75 ± 0.20a** | 57.73 ± 0.05b** |
| Acidity (NaOH/kg) | 142.61 ± 0.03** | 140.63 ± 0.10** | 119.45 ± 0.20** |

*Note*. Data = Means ± Standard deviations; Symbol ** = The observed values do not meet the Indonesian National Standard; Different letters indicate significant differences in values ($P$<0.01) as determined by the least significant difference test

conducted by Al-Ghamdi et al. (2019) and Pasias et al. (2018), indicating that hot tropical weather can elevate HMF content in honey within the hive. Despite these findings, the HMF concentration detected in the investigated honey is demonstrably lower when compared to honey produced in other tropical regions, such as Brazil that range from 2.61 to 3.81 mg/kg (dos Santos Scholz et al., 2020) and from 2.0 to 4.4 mg/kg (da S. Sant'ana et al., 2020).

The presence of HMF in honey can have negative and positive effects on human health. Negative effects include genotoxic, mutagenic, organotoxic, and enzyme-inhibiting properties, while positive effects encompass antioxidant, anti-allergic, anti-inflammatory, anti-hypoxic, and anti-hyperuricemia effects (Shapla et al., 2018). Analysis of HMF content in all honey samples employed within this investigation suggests the honey to be fresh and suitable for consumption.

**Sugar Content**

The sugars found in honey are monosaccharides and disaccharides (Chua & Adnan, 2014). This investigation focuses on quantifying monosaccharides within the sample, specifically those identified as reducing sugars. Quantification is performed by converting them to a glucose equivalent. Additionally, the analysis encompasses the disaccharide sugar sucrose.

The one-way ANOVA conducted in this study demonstrated a statistically significant effect ($P<0.01$) of both honey harvest time and the age of *A. crassicarpa*, the primary bee forage source, as well as their interaction, on the glucose content within the honey samples. LSD tests reveal a significant difference in glucose content between honey harvested at 14 and 21 days compared to honey harvested at 30 days ($P<0.01$) (Table 1). Honey samples collected from hives in the 8-month age group exhibited the highest glucose concentration (66.56% w/w). This value satisfies the established honey quality criterion for minimum reducing sugar content, set at 65% w/w.

The one-way ANOVA for sucrose content in honey also indicates a significant influence of harvest time, the age of *A. crassicarpa*, and their interaction. The lowest sucrose concentrations were observed in all honey samples harvested at 30 days. The optimal combination for sucrose content is a harvest time of 30 days with *A. crassicarpa* age of 8 months. The LSD test results indicate a significant difference between the 30-day harvest time and 8-month-old age combination compared to other combinations ($P<0.01$). Honey harvested 30 days from both the 8- and 18-month-old complied with the SNI 8664-2018 standard for sucrose content, which specifies a maximum level of 5% w/w sucrose in honey. Sucrose is a crucial parameter in testing honey's authenticity and maturity level. A high sucrose content in honey may indicate adulteration by adding sugarcane or beet sugar or prolonged feeding of honey bees with artificial substances such as syrup (Escuredo et al., 2013; Puscas et al., 2013).

The glucose and sucrose levels in honey samples display an opposing pattern, as the average glucose concentrations during the 30-day harvest surpass those during the 14 and 21-day harvests across all age groups of plants. In contrast, sucrose values are lower during the 30-day harvest. The extended duration of honey harvest is hypothesized to contribute to the observed transformation of sucrose into glucose. This proposition is in line with Boussaid et al. (2018) assertion that prolonged harvest times lead to heightened diastase enzyme activity, facilitating the conversion of disaccharides into monosaccharides. The composition of glucose and sucrose in honey is not solely influenced by harvest time but also by climatic conditions and nectar sources (Chua & Adnan, 2014; Escuredo et al., 2014; Juan-Borrás et al., 2014; Tornuk et al., 2013; Vranić et al., 2017). Monofloral honey derived from acacia plant nectar sources generally exhibits elevated sucrose levels compared to other nectar sources (Can et al., 2015; Juan-Borrás et al., 2014). The varying ages of *A. crassicarpa* plants lead to fluctuations in glucose and sucrose concentrations in the honey produced at that location, likely attributed to the distinct sugar compositions of nectar produced by these plants. However, currently, no research is available addressing the nectar composition produced by *A. crassicarpa* plants.

**Moisture Content**

Moisture content is a crucial quality parameter of honey, as it influences viscosity, specific gravity, taste, fermentation rate, and crystallization rate (Escuredo et al., 2013; Pasias et al., 2018; Wu et al., 2022). Excessively low moisture content can lead to caramelization in honey. In contrast, excessively high moisture content can result in fermentation and the formation of acetic acid, thereby increasing the likelihood of honey spoilage during storage (Boussaid et al., 2018; Kek et al., 2018).

This study revealed that the harvest time, age, and their interaction significantly influenced honey's moisture content ($P<0.01$). This study revealed that age significantly influenced honey's moisture content ($P<0.01$). LSD analysis further indicated a significant difference in honey moisture content between 30-day and 14- and 21-day harvests. However, there was no significant difference between the 14- and 21-day harvests ($P>0.05$) across all age groups of *A. crassicarpa*. Regarding the comparison of moisture content between the ages of *A. crassicarpa* for all harvest times, differences are only detected between the 3- and 18-month ages.

The moisture content in honey samples decreases with the increasing harvest time. The lowest moisture content is observed in honey harvested at 30 days across all age groups of plants. However, based on the results of the one-sample *t*-test, the honey harvested at 30 days from *A. crassicarpa* plants aged 3 and 18 months is the one that meets the Indonesian national honey quality standards, specifically below 22% w/w. Prolonged harvest time provides bees with additional time to decrease the water

content in the hive, resulting in more mature honey. This observation is consistent with the findings of do Nascimento et al. (2018) as well as Taha and AL-Kahtani (2020), who reported that honey moisture content is affected by harvest time and honey maturity level. The process of water content reduction within the hive occurs through active evaporation by worker bees, achieved by vibrating their wings (Abou-Shaara et al., 2017; Eyer et al., 2016).

The moisture content of honey harvested at 14 and 21 days is still relatively high, especially in the 3- and 8-month age groups, ranging from 23.4 to 26.4% w/w. These values are nearly identical to the findings of Suhesti, Roni, et al. (2023) for the moisture content of *A. mellifera* honey harvested at 14 days in a different location in Riau Province, Indonesia, measuring 26.73% w/w.

**Acidity Level**

An analysis of the acidity levels in all honey revealed values exceeding the maximum permissible limits established by SNI 8664:2018 (Badan Standardisasi Nasional, 2018). According to SNI 8664:2018, the maximum allowable acidity in honey is 50 milliequivalents sodium hydroxide (NaOH)/kg. This finding suggests that the investigated honey may not comply with current quality regulations. One-way ANOVA followed by LSD analysis indicates that harvest time, the age of *A. crassicarpa*, and their interactions significantly affect the acidity of honey, and acidity values differ among all harvest times and ages of *A. crassicarpa* in honey ($P<0.01$) (Table 1). There is a tendency for the acidity of honey to decrease with a longer harvest time, while an increase in the age of the plant as a nectar source tends to result in higher honey acidity.

The influence of harvest time on honey acidity is presumed to be due to the honey's maturation level. Da Silva et al. (2016) stated that an extended harvest time increases honey maturation. The dominant acid in honey is gluconic acid, formed through the oxidation of glucose during honey maturation by bees (Karabagias et al., 2014). This study investigates the potential correlation between the age of *A. crassicarpa* and the resulting honey acidity produced by *A. mellifera* in the local region. It hypothesizes that as *A. crassicarpa* matures, the extrafloral nectar secreted from its leaf bases exhibits an increase in acidity, which may, in turn, influence the honey produced by *A. mellifera*. While the correlation between nectar sugar composition and honey acidity is a well-established concept, further study is necessary to explore the potential influence of the peatland environment on honey acidity. Since the acidic nature of peat swamp environments, a characteristic habitat for *A. crassicarpa* may contribute to the observed acidity levels in the honey produced from its nectar (Suhesti, Zalizar, et al., 2023). Studies by Erniaty et al. (2023) support this hypothesis, demonstrating the low pH and high acidity typical of peat swamp ecosystems.

Acidity in honey imparts chemical and sensory characteristics, influencing honey's taste and consumer preferences (Al-Ghamdi

et al., 2019; dos Santos Scholz et al., 2020; Suhesti, Roni, et al., 2023). Furthermore, the acidity level in honey can serve as an indicator that the honey has undergone fermentation (Boussaid et al., 2018), which can occur rapidly in conditions with high moisture content. However, all honey samples in this study are freshly harvested, so that the elevated acidity levels can be attributed to factors other than fermentation. Ananias et al. (2013) stated that honey with high acidity values but without signs of fermentation cannot be considered of lower quality, as factors influencing acidity include environmental conditions, harvest time, nectar source plants, and climate (da Silva et al., 2016; dos Santos Scholz et al., 2020). The acidity in honey may also indicate the presence of antioxidants often associated with ascorbic acid or vitamin C (Pribadi & Wiratmoko, 2023). A study by Handayani et al. (2022) investigated *A. mellifera* honey produced from *A. crassicarpa* nectar in Siak Regency, Indonesia. Their research demonstrated that the honey possessed antioxidant activity, with a measured value of 21,103.74 µg/ml.

Additionally, the study identified the presence of various secondary metabolites within the honey, including alkaloids, phenolics, flavonoids, terpenoids, saponins, and tannins. These compounds suggest that honey has the potential to be developed into a new functional beverage when combined with other ingredients, offering enhanced health benefits through their antioxidant, anti-inflammatory, and antimicrobial properties (Maulida et al., 2024).

## CONCLUSION

The results indicated significant influences of harvest time, *A. crassicarpa* age, and the interaction between harvest time and *A. crassicarpa* age on various parameters, such as enzymatic activity, HMF content, sugar composition, moisture content, and acidity. Based on the analysis, the research findings indicate a significant interaction between the harvest time of 30 days and the age of *A. crassicarpa* plants, particularly those aged 8 and 18 months. This interaction resulted in the production of honey with the highest levels of HMF and glucose, as well as the lowest moisture and sucrose content. Importantly, these parameters met the specified national quality standards. Future research should focus on elucidating the chemical composition of *A. crassicarpa* nectar and exploring the broader implications of honey composition for various honeybee species. The challenging aspects of this study involve addressing the complex interplay between environmental factors, harvest time, and plant age, which may contribute to the variability in honey composition, especially for acidity level.

## ACKNOWLEDGMENTS

We are grateful to the Ministry of Education, Culture, Research, and Technology, Directorate General of Higher Education of the Republic of Indonesia, for funding this research under the Doctoral Dissertation Research Grant scheme, grant number 183/E5/PG.02.00.PL/2023; 013/SP2H/PT/LL7/2023.

## REFERENCES


Abou-Shaara, H. F., Owayss, A. A., Ibrahim, Y. Y., & Basuny, N. K. (2017). A review of impacts of temperature and relative humidity on various activities of honey bees. *Insectes Sociaux*, *64*, 455–463. https://doi.org/10.1007/s00040-017-0573-8

Al-Ghamdi, A., Mohammed, S. E. A., Ansari, M. J., & Adgaba, N. (2019). Comparison of physicochemical properties and effects of heating regimes on stored *Apis mellifera* and *Apis florea* honey. *Saudi Journal of Biological Sciences*, *26*(4), 845–848. https://doi.org/10.1016/j.sjbs.2017.06.002

Alvarez-Suarez, J. M., Giampieri, F., Brenciani, A., Mazzoni, L., Gasparrini, M., González-Paramás, A. M., Santos-Buelga, C., Morroni, G., Simoni, S., Forbes-Hernández, T. Y., Afrin, S., Giovanetti, E., & Battino, M. (2018). *Apis mellifera vs Melipona beecheii* Cuban polifloral honeys: A comparison based on their physicochemical parameters, chemical composition and biological properties. *LWT*, *87*, 272–279. https://doi.org/10.1016/j.lwt.2017.08.079

Ananias, K. R., de Melo, A. A. M., & de Moura, C. J. (2013). Analysis of moisture content, acidity and contamination by yeast and molds in *Apis mellifera* L. honey from central Brazil. *Brazilian Journal of Microbiology*, *44*(3), 679–683. https://doi.org/10.1590/S1517-83822013000300003

Badan Standardisasi Nasional. (2018). *SNI 8664-2018: Madu* [SNI 8664-2018: Honey]. https://pesta.bsn.go.id/produk/detail/13137-sni86642018

Baroni, M. V., Podio, N. S., Badini, R. G., Inga, M., Ostera, H. A., Cagnoni, M., Gautier, E. A., García, P. P., Hoogewerff, J., & Wunderlin, D. A. (2015). Linking soil, water, and honey composition to assess the geographical origin of Argentinean honey by multielemental and isotopic analyses. *Journal of Agricultural and Food Chemistry*, *63*(18), 4638–4645. https://doi.org/10.1021/jf5060112

Bell, A. R., & Grainger, M. N. C. (2023). Accelerated loss of diastase in mānuka honey: Investigation of mānuka specific compounds. *Food Chemistry*, *426*, 136614. https://doi.org/10.1016/j.foodchem.2023.136614

Berhe, A., Tesfaye, E., & Terle, D. (2018). Evaluation of physicochemical properties of honey bees (*Apis mellifera*) in Godere woreda, Gambella, Ethiopia. *American Journal of Food Science and Technology*, *6*(1), 50–56. https://doi.org/10.12691/ajfst-6-1-8

Boussaid, A., Chouaibi, M., Rezig, L., Hellal, R., Donsì, F., Ferrari, G., & Hamdi, S. (2018). Physicochemical and bioactive properties of six honey samples from various floral origins from Tunisia. *Arabian Journal of Chemistry*, *11*(2), 265–274. https://doi.org/10.1016/j.arabjc.2014.08.011

Can, Z., Yildiz, O., Sahin, H., Turumtay, E. A., Silici, S., & Kolayli, S. (2015). An investigation of Turkish honeys: Their physico-chemical properties, antioxidant capacities and phenolic profiles. *Food Chemistry*, *180*, 133–141. https://doi.org/10.1016/j.foodchem.2015.02.024

Chua, L. S., & Adnan, N. A. (2014). Biochemical and nutritional component of selected honey samples. *Acta Scientiarum Polonorum, Technologia Alimentaria*, *13*(2), 169–179.

Czipa, N., Phillips, C. J. C., & Kovács, B. (2019). Composition of acacia honeys following processing, storage and adulteration. *Journal of Food Science and Technology*, *56*(3), 1245–1255. https://doi.org/10.1007/s13197-019-03587-y

da S. Sant'ana, R., de Carvalho, C. A. L., Oda-Souza, M., de A. Souza, B., & de S. Dias, F. (2020). Characterization of honey of stingless bees from the Brazilian semi-arid region. *Food Chemistry*, *327*, 127041. https://doi.org/10.1016/j.foodchem.2020.127041

da Silva, P. M., Gauche, C., Gonzaga, L. V., Costa, A. C. O., & Fett, R. (2016). Honey: Chemical composition, stability and authenticity. *Food Chemistry*, *196*, 309–323. https://doi.org/10.1016/j.foodchem.2015.09.051

De-Melo, A. A. M., de Almeida-Muradian, L. B., Sancho, M. T., & Pascual-Maté, A. (2018).

Composición y propiedades de la miel de *Apis mellifera*: Una revisión [Composition and properties of *Apis mellifera* honey: A review]. *Journal of Apicultural Research*, *57*(1), 5–37. https://doi.org/10.1080/00218839.2017.1338444

do Nascimento, K. S., Sattler, J. A. G., Macedo, L. F. L., González, C. V. S., de Melo, I. L. P., da Silva Araújo, E., Granato, D., Sattler, A., & de Almeida-Muradian, L. B. (2018). Phenolic compounds, antioxidant capacity and physicochemical properties of Brazilian *Apis mellifera* honeys. *LWT*, *91*, 85–94. https://doi.org/10.1016/j.lwt.2018.01.016

dos Santos Scholz, M. B., Júnior, A. Q., Delamuta, B. H., Nakamura, J. M., Baudraz, M. C., Reis, M. O., Kato, T., Pedrão, M. R., Dias, L. F., dos Santos, D. T. R., Kitzberger, C. S. G., & Bianchini, F. P. (2020). Indication of the geographical origin of honey using its physicochemical characteristics and multivariate analysis. *Journal of Food Science and Technology*, *57*, 1896–1903. https://doi.org/10.1007/s13197-019-04225-3

Erban, T., Shcherbachenko, E., Talacko, P., & Harant, K. (2021). A single honey proteome dataset for identifying adulteration by foreign amylases and mining various protein markers natural to honey. *Journal of Proteomics*, *239*, 104157. https://doi.org/https://doi.org/10.1016/j.jprot.2021.104157

Erniaty, E., Gumiri, S., Ardianor, A., Haryono, A., Yulintine, Y., & Masliani, M. (2023). Analysis of water physical, chemical and biological properties in different peat swamp forests converted to rice fields. In *IOP Conference Series: Earth and Environmental Science* (Vol. 1153, No. 1, p. 012026). IOP Publishing. https://doi.org/10.1088/1755-1315/1153/1/012026

Escuredo, O., Dobre, I., Fernández-González, M., & Seijo, M. C. (2014). Contribution of botanical origin and sugar composition of honeys on the crystallization phenomenon. *Food Chemistry*, *149*, 84–90. https://doi.org/10.1016/j.foodchem.2013.10.097

Escuredo, O., Míguez, M., Fernández-González, M., & Seijo, M. C. (2013). Nutritional value and antioxidant activity of honeys produced in a European Atlantic area. *Food Chemistry*, *138*(2–3), 851–856. https://doi.org/10.1016/j.foodchem.2012.11.015

Eyer, M., Neumann, P., & Dietemann, V. (2016). A look into the cell: Honey storage in honey bees, *Apis mellifera*. *PLOS One*, *11*(8), e0161059. https://doi.org/10.1371/journal.pone.0161059

Ghramh, H. A., Alrumman, S. A., Ahmad, I., Kalam, A., Elbehairi, S. E. I., Alfaify, A. M., Mohammed, M. E. A., Al-Sehemi, A. G., Alfaifi, M., Al-Shehri, B. M., Alshareef, R. M. H., Alaerjani, W. M. A., & Khan, K. A. (2023). Chemical characterization of honey and its effect (alone as well as with synthesized silver nanoparticles) on microbial pathogens' and human cancer cell lines' growth. *Nutrients*, *15*(3), 684. https://doi.org/10.3390/nu15030684

Hailu, D., & Belay, A. (2020). Melissopalynology and antioxidant properties used to differentiate *Schefflera abyssinica* and polyfloral honey. *PLOS One*, *15*(10), e0240868. https://doi.org/10.1371/journal.pone.0240868

Handayani, T. H., Budiman, M. A., Amalia, R. L. R., Pribadi, A., Elfitra, R. R., & Ferdian, P. R. (2022). Aktivitas antioksidan, total fenolik, dan total flavonoid madu *Apis mellifera* dari hutan akasia (*Accacia crassicarpa*) Riau, Indonesia dengan beberapa perlakuan pengeringan [Antioxidant activity, total phenolics, and total flavonoids of *Apis mellifera* honey from acacia (*Accacia crassicarpa*) forests in Riau, Indonesia with several drying treatments]. *Jurnal Biologi Indonesia*, *18*(2), 231–243. https://doi.org/10.47349/jbi/18022022/231

Juan-Borrás, M., Domenech, E., Hellebrandova, M., & Escriche, I. (2014). Effect of country origin on physicochemical, sugar and volatile composition of acacia, sunflower and tilia honeys. *Food Research International*, *60*, 86–94. https://doi.org/10.1016/j.foodres.2013.11.045

Karabagias, I. K., Badeka, A., Kontakos, S., Karabournioti, S., & Kontominas, M. G. (2014). Characterisation and classification of Greek pine honeys according to their geographical origin based on volatiles, physicochemical

parameters and chemometrics. *Food Chemistry*, *146*, 548–557. https://doi.org/10.1016/j.foodchem.2013.09.105

Kek, S. P., Chin, N. L., Yusof, Y. A., Tan, S. W., & Chua, L. S. (2018). Classification of entomological origin of honey based on its physicochemical and antioxidant properties. *International Journal of Food Properties*, *20*(sup3), S2723–S2738. https://doi.org/10.1080/10942912.2017.1359185

Lewkowski, O., Mureşan, C. I., Dobritzsch, D., Fuszard, M., & Erler, S. (2019). The effect of diet on the composition and stability of proteins secreted by honey bees in honey. *Insects*, *10*(9), 282. https://doi.org/10.3390/insects10090282

Maulida, I. D., Al Marsam, M. R., Purnama, I., & Mutamima, A. (2024). A novel beverage with functional potential incorporating cascara (*Coffea arabica*), roselle (*Hibiscus sabdariffa*), and red ginger (*Zingiber officinale* Rosc. var. *rubrum*) extracts: Chemical properties and sensory evaluation. *Discover Food*, *4*, 94. https://doi.org/10.1007/s44187-024-00180-x

Oroian, M., & Sorina, R. (2017). Honey authentication based on physicochemical parameters and phenolic compounds. *Computers and Electronics in Agriculture*, *138*, 148–156. https://doi.org/10.1016/j.compag.2017.04.020

Pasias, I. N., Kiriakou, I. K., Kaitatzis, A., Koutelidakis, A. E., & Proestos, C. (2018). Effect of late harvest and floral origin on honey antibacterial properties and quality parameters. *Food Chemistry*, *242*, 513–518. https://doi.org/10.1016/j.foodchem.2017.09.083

Pribadi, A., & Purnomo. (2013). *Agroforestry sorghum (*Sorghum *spp.) pada HTI* Acacia crassicarpa *sebagai sumber pakan lebah* Apis cerana *di Provinsi Riau untuk mendukung budidaya lebah madu* [Agroforestry sorghum (*Sorghum* spp.) on IPF *Acacia crassicarpa* as a source of feed for *Apis cerana* bees in Riau Province to support honey bee cultivation]. In D. P. Kuswantoro, T. S. Widyaningsih, E. Fauziyah, & R. Rachmawati (Eds.), Prosiding Seminar Nasional Agroforestri 2013: Agroforestri untuk Pangan dan Lingkungan yang Lebih Baik (pp. 36-41). Balai Penelitian Teknologi Agroforestry, Fakultas Pertanian Universitas Brawijaya, World Agroforestry Centre (ICRAF), and Masyarakat Agroforestri Indonesia.

Pribadi, A., & Wiratmoko, M. D. E. (2023). Karakteristik fisikokimia madu *Heterotrigona itama* asal Provinsi Riau [Physicochemictry characteristics of *Heterotrigona itama* honey from Riau Province]. *Wahana Forestra: Jurnal Kehutanan*, *18*(2), 105-120. https://doi.org/10.31849/forestra.v18i2.11107

Purwanto, D. B., Kuntadi., Adalina, Y., Meilin, A., Raffiudin, R., & Sahlan, M. (2024). Evaluation of honey production for industry purposes, the case of beekeeping with *Apis mellifera* in Indonesia after the COVID-19 pandemic. *International Journal of Social Service and Research*, *4*(3), 702-715. https://doi.org/10.46799/ijssr.v4i03.719

Puscas, A., Hosu, A., & Cimpoiu, C. (2013). Application of a newly developed and validated high-performance thin-layer chromatographic method to control honey adulteration. *Journal of Chromatography A*, *1272*, 132–135. https://doi.org/10.1016/j.chroma.2012.11.064

Radtke, J., & Lichtenberg-Kraag, B. (2018). Long-term changes in naturally produced honey depending on processing and temperature. *Journal of Apicultural Research*, *57*(5), 615–626. https://doi.org/10.1080/00218839.2018.1494893

Sajid, M., Yasmin, T., Asad, F., & Qamer, S. (2019). Changes in HMF content and diastase activity in honey after heating treatment. *Pure and Applied Biology*, *8*(2), 1668–1674. https://doi.org/10.19045/BSPAB.2019.80109

Shapla, U. M., Solayman, M., Alam, N., Khalil, M. I., & Gan, S. H. (2018). 5-hydroxymethylfurfural (HMF) levels in honey and other food products: Effects on bees and human health. *Chemistry Central Journal*, *12*, 35. https://doi.org/10.1186/s13065-018-0408-3

Siddiqui, A. J., Musharraf, S. G., Choudhary, M. I., & Atta-ur-Rahman. (2017). Application of analytical methods in authentication and adulteration of honey. *Food Chemistry*,

*217*, 687–698. https://doi.org/10.1016/j.foodchem.2016.09.001

Suhesti, E., Roni, Y., Yanti, R. N., Ningsih, A. T., & Hadinoto. (2023). Kualitas dan preferensi konsumen terhadap madu lebah *Apis mellifera* L. dan *Apis dorsata* F. [Quality and consumer preferences for *Apis mellifera* L. and *Apis dorsata* F. honey]. *Jurnal Penelitian Hasil Hutan*, *41*(2), 87–96. https://doi.org/10.55981/jphh.2023.766

Suhesti, E., Zalizar, L., Triwanto, J., Ervayendri, E., Setyobudi, R. H., Waskitho, N. T., Ibrahim, J. T., Maftuchah, M., Hadinoto, H., Vincēviča-Gaile, Z., Tonda, R., Angara, A., Hartono, R., Lestari, S. U., & Pakarti, T. A. (2023). Quality assessment on honey produced from six months old *Acacia crassicarpa*. In *E3S Web of Conferences: The 3rd International Conference on Natural Resources and Life Sciences 2020* (Vol. 374, p. 00012). *EDP Sciences*. https://doi.org/10.1051/e3sconf/202337400012

Taha, E.-K. A., & AL-Kahtani, S. N. (2020). The relationship between comb age and performance of honey bee (*Apis mellifera*) colonies. *Saudi Journal of Biological Sciences*, *27*(1), 30–34. https://doi.org/10.1016/j.sjbs.2019.04.005

Taha, E.-K. A., Al-Kahtani, S., & Taha, R. (2021). Comparison of the physicochemical characteristics of sidr (*Ziziphus* spp.) honey produced by *Apis florea* F. and *Apis mellifera* L. *Journal of Apicultural Research*, *60*(3), 470–477. https://doi.org/10.1080/00218839.2020.1746036

Tong, L., Nieh, J. C., & Tosi, S. (2019). Combined nutritional stress and a new systemic pesticide (flupyradifurone, Sivanto®) reduce bee survival, food consumption, flight success, and thermoregulation. *Chemosphere*, *237*, 124408. https://doi.org/10.1016/j.chemosphere.2019.124408

Tornuk, F., Karaman, S., Ozturk, I., Toker, O. S., Tastemur, B., Sagdic, O., Dogan, M., & Kayacier, A. (2013). Quality characterization of artisanal and retail Turkish blossom honeys: Determination of physicochemical, microbiological, bioactive properties and aroma profile. *Industrial Crops and Products*, *46*, 124–131. https://doi.org/10.1016/j.indcrop.2012.12.042

Viteri, R., Zacconi, F., Montenegro, G., & Giordano, A. (2021). Bioactive compounds in *Apis mellifera* monofloral honeys. *Journal of Food Science*, *86*(5), 1552–1582. https://doi.org/10.1111/1750-3841.15706

Vranić, D., Petronijević, R., Stojanović, J. Đ., Korićanac, V., Milijašević, J. B., & Milijašević, M. (2017). Physicochemical properties of honey from Serbia in the period 2014-2016. In *IOP Conference Series: Earth and Environmental Science* (Vol. 85, No. 1, p. 012058). IOP Publishing. https://doi.org/10.1088/1755-1315/85/1/012058

Wu, M.-C., Wu, C.-Y., Klaithin, K., Tiong, K. K., & Peng, C.-C. (2022). Effect of harvest time span on physicochemical properties, antioxidant, antimicrobial, and anti-inflammatory activities of Meliponinae honey. *Journal of the Science of Food and Agriculture*, *102*(13), 5750–5758. https://doi.org/10.1002/jsfa.11924

Pertanika J. Trop. Agri. Sci. 47 (4): 1221 - 1243 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

*Review Article*

# A Review of Pangasiid Catfish Genomics for Conservation and Aquaculture: Current Status and Way Forward

**Siti Amalia Aisyah Abdul-Halim[1], Yuzine Esa[1,2,3]*, Thuy-Yen Duong[6], Fadhil Syukri[1], Heera Rajandas[4], Sivachandran Parimannan[4] and Siti Azizah Mohd-Nor[5]**

[1]*Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

[2]*International Institute of Aquaculture and Aquatic Sciences, Universiti Putra Malaysia, 70150 Port Dickson, Malaysia*

[3]*Department of Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, Campus C Jalan Mulyorejo, Surabaya 60115 East Java, Indonesia*

[4]*Centre of Excellence for Omics-Driven Computational Biodiscovery, Faculty of Applied Sciences, AIMST University, 08100 Bedong, Malaysia*

[5]*Institute of Climate Adaptation and Marine Biotechnology, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia*

[6]*College of Aquaculture and Fisheries, Can Tho University, 3/2 Street, 94115 Can Tho, Vietnam*

## ABSTRACT

Due to its ecological and economic importance, the family Pangasiidae has been the focus of considerable biological and genetic research. This family includes thirty species of catfish distributed among four genera: *Helicophagus*, *Pangasianodon*, *Pangasius*, and *Pseudolais* within the order Siluriformes. In recent years, genomic-scale data for a wide range of aquatic species have been more easily available and accessible through next-generation sequencing. Draft genome sequences of numerous fishes have been the basis for many downstream investigations. However, although genomic-based technologies allow for the collection of robust data, there is a dearth of research on pangasiid catfishes, which could potentially enhance fisheries' management and aquaculture. To realise its aquaculture potential and strategise the conservation of this family, fundamental knowledge of its biology, genetics, and genomics, among others, is critical. This review focuses on the current molecular knowledge of the family Pangasiidae, the remaining gaps, and the challenges faced in closing these gaps.

*Keywords*: Aquaculture, conservation, genomics, mitogenome, Pangasiidae

ARTICLE INFO

*Article history:*
Received: 20 February 2024
Accepted: 22 April 2024
Published: 25 October 2024

DOI: https://doi.org/10.47836/pjtas.47.4.11

E-mail addresses:
ameliaaisya245@gmail.com (Siti Amalia Aisyah Abdul-Halim)
yuzine@upm.edu.my (Yuzine Esa)
thuyyen@ctu.edu.vn (Thuy-Yen Duong)
fadhil@upm.edu.my (Fadhil Syukri)
heraadaas@gmail.com (Heera Rajandas)
sivachandran@aimst.edu.my (Sivachandran Parimannan)
s.azizah@umt.edu.my (Siti Azizah Mohd-Nor)
*Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

## AN OVERVIEW OF FAMILY PANGASIIDAE

The family Pangasiidae has been the subject of considerable biological and genetic research due to its ecological and economic significance in aquaculture. A tremendous diversity of this family is found in Southeast Asia (Vietnam, Thailand, Laos, Cambodia, Myanmar, Peninsula Malaysia, Borneo, Java, and Sumatra), with additional species in India and China (Fricke et al., 2023; Froese & Pauly, 2024; Kottelat, 2013; Parenti & Lim, 2005; Roberts & Vidthayanon, 1991). Members of this family are usually found in freshwater, but several species inhabit brackish and marine environments as part of their life cycles (Gustiano, 2009; Roberts & Vidthayanon, 1991; Vu et al., 2020). In Malaysia, species of this family are collectively known as 'Patin'.

The pangasiids represent a diverse species composition of 30 valid species within four genera (Figure 1): *Helicophagus* (3 species), *Pangasianodon* (2 species), *Pangasius* (23 species), and *Pseudolais* (2 species) (Fricke et al., 2023; Froese & Pauly, 2024; Gustiano, 2009; Kottelat, 2013). Adult pangasiid catfishes can be identified by their laterally compressed bodies, two sets of barbels (maxillary and mandibular), a long anal fin, a short dorsal fin with strong spines, a small adipose fin that is never confluent with the caudal fin, and smooth skin (Froese & Pauly, 2024; Gustiano, 2009; Roberts & Vidthayanon, 1991). The dominant genus, *Pangasius* (76%), occurs in the Indo-Malay Archipelago (IMA) and Indo-China hotspot regions of Peninsula Malaysia, Borneo, Indonesia, Thailand, Cambodia, and Vietnam. These distribution ranges had been mapped based on combined pieces of literature (Froese & Pauly, 2024; Gustiano, 2009; Inger & Chin, 1962; Kottelat, 2013; Roberts & Vidthayanon, 1991). This genus is characterised by the presence of long maxillary barbels, six-branched pelvic-fin rays, and pectoral and adipose fins that are relatively robust (Froese & Pauly, 2024; Gustiano, 2009; Roberts & Vidthayanon, 1991).

Members of the pangasiid family are mainly distributed in lakes and river basins (Figure 1). Peninsular Malaysia (Perak and Pahang Rivers), Borneo (Batang Rajang, Kinabatangan, Kayan, Berau, Mahakam, Barito, Kahayan and Kapuas Rivers) and Sumatra (Indragiri, Musi, Batang Hari and Way Rarem Rivers) harbour three genera including *Helicophagus*, *Pangasius*, and *Pseudolais*. The Indochina regions (Chao Phraya, Mae Klong, Hue, and Mekong Rivers) have all four genera, while Java (Ciliwung, Solo, and Brantas Rivers) has only two genera (Figure 1). Their conservation status is considerable. Several are commonly found throughout the pangasiid home range, while others are globally or locally threatened.

Species distributions of pangasiids have been extensively studied since the 1990s, thanks to a multitude of sampling initiatives across Southeast Asia. In Indo-China, 13 species were inhabit the Mekong River, Chao Phraya River, and Mae Klong River (*Helicophagus leptorhynchus*,

*Pangasianodon gigas*, *Pangasianodon hypophthalmus*, *Pangasius bocourti*, *Pangasius conchophilus*, *Pangasius elongates*, *Pangasius krempfi*, *Pangasius larnaudii*, *Pangasius macronema*, *Pangasius mekongensis*, *Pangasius sanitwongsei*, *Pseudolais micronemus*, and *Pseudolais pleurotaenia*) (Figure 1) (Fricke et al., 2023; Froese & Pauly, 2024; Roberts & Baird, 1995; Roberts & Vidthayanon, 1991). The Malaysian waters are home to several catfish species. Eight species are native to Malaysian rivers. Three of these native species (*Helicophagus waandersii*, *P. micronemus*, and *Pangasius nasutus*) inhabit the major rivers of Peninsula Malaysia, the Pahang River, while *P. micronemus* can also be found in Perak and the major Borneo rivers of Batang Rajang, and Kinabatangan (Baharuddin, 2016; Gustiano, 2009; Roberts & Vidthayanon, 1991). Moreover, *P. nasutus* was also reported in Batang Rajang (Parenti & Lim, 2005). In addition, five more species occur in the Batang Rajang and Kinabatangan rivers (*Pangasius polyuranodon*, *Pangasius humeralis*, *Pangasius lithostoma*, *Pangasius kinabatanganensis*, and *Pangasius sabahensis*). The latter four are believed to be endemic to Borneo (Gustiano & Pouyaud, 2005; Kottelat, 2013; Parenti & Lim, 2005). It is not unexpected as the freshwater fish fauna of northwestern Borneo, which includes Sarawak, Sabah, and Brunei, is known for its high degree of endemism (Parenti, 1996; Parenti & Lim, 2005).

Inland fisheries in Borneo are important, mostly along rivers, with the rest from lakes, reservoirs, and flood plains (Inger & Chin, 1962; Parenti & Lim, 2005). They have a significant impact on the rural community's economic well-being by creating jobs and investment opportunities, apart from providing abundant sources of protein. However, although these inland fish resources offer low-cost sources of food and nutrition for the people, they make very little contribution to total fishery production and export values due to their low production (Chong et al., 2010; Inger & Chin, 1962).

Despite its importance, this family has relatively limited genomic information, acknowledged as one of the important current perspectives for accelerating the aquaculture industry. A few reviews have been documented, mainly focused on biological diversity, taxonomy and systematics, aquaculture and phylogeographic relationships (Legendre, 1998; Pouyaud et al., 1998; Roberts & Vidthayanon, 1991). Given the present scenario of rapid population declines, coupled with the higher demand for affordable fish protein worldwide, a holistic understanding of the pangasiid family is necessary. While previous studies have provided important knowledge for the first step towards addressing this problem, more work needs to be done, particularly in genomics. This review could serve as a reference for future research efforts in conserving the pangasiid family in the biodiversity hotspots of Southeast Asia based on molecular approaches.

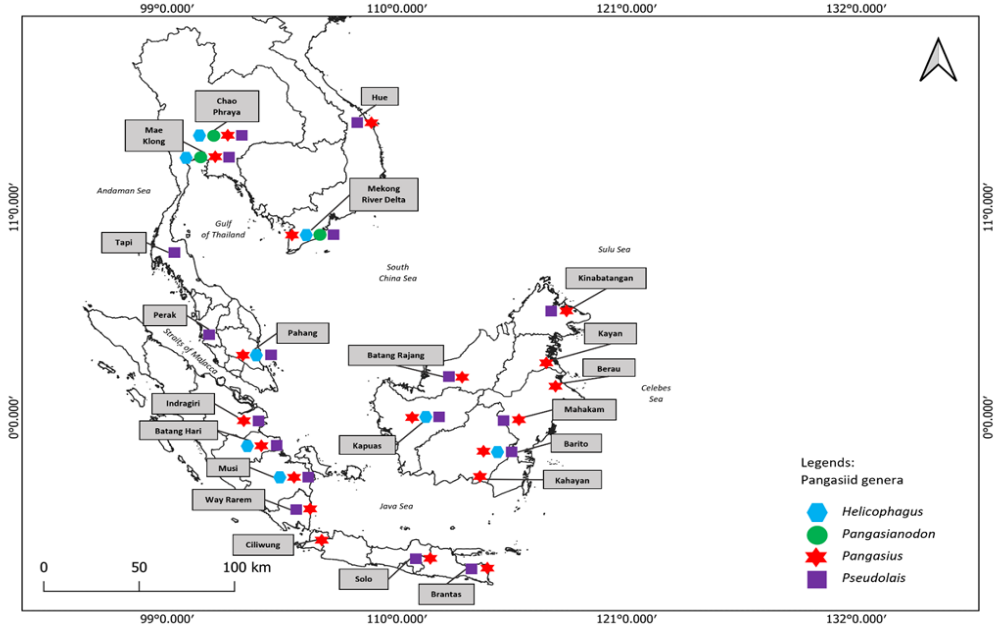



*Figure 1*. Range distribution of catfish family of Pangasiidae based on genera throughout the Southeast Asia river basins regions

*Note*. The box shape indicates river location; The shapes colour indicates genera of family Pangasiidae

## THE INTERNATIONAL UNION FOR CONSERVATION OF NATURE (IUCN) RED LIST STATUS OF PANGASIID SPECIES

Several pangasiids are common throughout their home range, while others are globally or locally threatened. Based on the IUCN Red List (IUCN, 2023), two species, *P. gigas* and *P. sanitwongsei*, are classified as Critically Endangered (CR) in the Mekong and Chao Phraya Rivers (Jondeung et al., 2007; Sriphairoj et al., 2007, 2018). *Pangasianodon hypophthalmus* is categorised as Endangered (EN), while *P. krempfi* is Vulnerable (VU) (Duong, Nguyen, et al., 2023; IUCN, 2023). In addition, *Pangasius rheophilus*, a native species from Kalimantan, Indonesia, has also been recently classified as Near Threatened (NT) (Gustiano & Pouyaud, 2005; IUCN, 2023). The common factors that explain the species declination in all these regions include over-harvesting/over-fishing, by-catch, habitat loss/degradation, habitat fragmentation, pollution, human disturbance, and endemism (Chong et al., 2010; Gustiano & Pouyaud, 2005; Inger & Chin, 1962; Parenti & Lim, 2005).

In the Malaysian scenario, *H. waandersii* (Patin Muncung), *P. nasutus* (Patin Buah), *P. kinabatanganensis*, and *P. polyuranodon* are classified as Moderately Threatened (MT), which is equivalent to Endangered (EN) in IUCN 2023 (Chong et al., 2010). The leading causes have been attributed to overharvesting and habitat degradation;

however, in the case of *P. kinabatanganensis*, it is believed to be due to overharvesting on this endemic species. According to Moyle and Leidy (1992), freshwater species that have adapted for life in large rivers or are endemic are in high danger of extinction. On the other hand, estuarine fishes are more likely to be threatened due to their reliance on freshwater inflows (Vu et al., 2020). Considering the threatened status of several species, a comprehensive understanding of several pertinent aspects of the pangasiids is critical. Given the broader implications of continuous habitat degradation to fish productivity, habitat conservation is tightly linked to fish conservation and should be considered an essential fishery management objective. However, it must go in parallel with an updated assessment of the species' conservation status.

## ADVANCES IN GENOMIC RESEARCH ON CATFISH SPECIES

The primary goal of genomics research is discovering how the genome functions. Fundamental genome information permits the identification of potential species for aquaculture (Liu, 2007) with desirable traits for production, strengthening resistance to diseases and improving growth rates, thus ensuring the long-term sustainability of aquaculture practices. Therefore, it is essential to understand and interrogate the genetic structure and organisation of a potential aquaculture species and its genomic variants to understand its functional properties (Lu & Luo, 2020; The Aquaculture Genomics, Genetics and Breeding Workshop et al., 2017; Yue & Wang, 2017). The freshwater aquaculture sector relies heavily on catfish for its numerous advantageous traits, making the study of their genome a priority (Jin et al., 2016; Kim et al., 2018; Wang, Su, et al., 2022). Several species have been farmed, and there are many candidate species for farming and breeding. Ongoing research endeavours have been initiated in the domain of genomic studies in catfish species, yielding noteworthy progress in this study area.

### Whole Genome Sequencing (WGS)

Knowledge of the whole genome of an organism, which covers all of its genetic material, including both the coding and non-coding regions, is the ultimate data to explore the full potential of a species for an aquaculture programme (Goodwin et al., 2016; Liu, 2007). By sequencing the complete catfish genome, researchers will have a holistic and deeper grasp of the genetic variation present in each fish. Whole genome sequencing of several catfish species has furnished some breakthroughs. The channel catfish (*Ictalurus punctatus*) (Jin et al., 2016; Liu et al., 2016) and the striped catfish (*P. hypophthalmus*) (Gao et al., 2021; Hai et al., 2022; Kim et al., 2018) genomes were among the first catfish genomes to be sequenced. These genome sequences have provided valuable information for researchers in various fields, including aquaculture and conservation (Jin et al., 2016; Liu, 2007; Lu & Luo, 2020; The Aquaculture Genomics, Genetics and

Breeding Workshop et al., 2017). WGS is increasingly becoming one of the most utilised applications in the next-generation sequencing (NGS) approach. Through this technology, researchers can acquire the most extensive understanding of genomic data and its related biological impacts (Goodwin et al., 2016; Liu, 2007).

These draft genome sequences have served as the basis for a plethora of investigations, such as resource assessment, resistance to disease, growth performances, sex determination, fisheries management, and environmental adaptation (Hai et al., 2022; Jin et al., 2016; Lu & Luo, 2020; The Aquaculture Genomics, Genetics and Breeding Workshop et al., 2017) in key aquatic species. For instance, the genome of Channel catfish (*I. punctatus*), the most cultured species in the U.S., has been widely investigated to understand evolution and important biological characteristics (Jin et al., 2016). Another species of catfish that has been widely explored for its whole genome sequence is *Ictalurus furcatus* (blue catfish) (Wang, Su, et al., 2022), which currently serves as a reference sequence for the study of other blue catfish for various purposes, i.e., gene expression, chromosome inversion, and single nucleotide polymorphism (SNP) identification (Jin et al., 2016; The Aquaculture Genomics, Genetics and Breeding Workshop et al., 2017; Wang, Montague, et al., 2022). However, despite numerous studies on catfish genomes (Table 1), research in pangasiid catfish remains limited.

Within the pangasiid catfishes, only the draft genome of the striped catfish, *P. hypophthalmus* (Gao et al., 2021; Hai et al., 2022; Kim et al., 2018) is available. The first draft genome of *P. hypophthalmus* was obtained using Illumina Miseq and Hiseq sequencing platforms (Kim et al., 2018) and was about 715.8 Mb with scaffold and contig N50 sizes of 14.28 Mb and 6 kb, respectively (Table 1). The striped catfish genome was predicted to consist of 28,600 protein-coding genes. However, in their study on *P. hypophthalmus*, the genome was based on only the Illumina short read. Chromosome-level genome assembly of the same species has been recently reported (Gao et al., 2021; Hai et al., 2022). Using a combination of Illumina short reads, Nanopore long reads and Hi-C data successfully improved the quality of the chromosome-level genome assembly of the striped catfish (Gao et al., 2021) (Table 1). Based on precise long-read HiFi sequencing data, Hai et al. (2022) reported the genome size to be 788.4 Mb, consisting of 381 scaffolds with an N50 length of 21.8 Mb. This latest assembly has a contig N50 of 14.7 Mb, which is 245- and 4.2-fold better than the previous Illumina (Kim et al., 2018) and Illumina-Nanopore-Hi-C-based versions (Gao et al., 2021), respectively. Such studies represent the groundwork for more in-depth genome research on the pangasiid catfishes.

**Transcriptome Resources**

It is not always possible to study the whole genome, particularly due to budget constraints. Another useful genomic

Table 1
*Current genomic information of catfish species*

| Species | Common name | Chromosome no. (2n) | Genome assembly | | Genome annotation | Sequencing platforms | References |
|---|---|---|---|---|---|---|---|
| | | | Genome size (Mb) | Scaffold N50 (Mb) | No. of protein-coding genes | | |
| *Pangasianodon hypophthalmus* | Striped catfish | 2n = 60 | 715.80 | 14.28 | 28,600 | Illumina Miseq and Hiseq | Kim et al. (2018) |
| | | | 731.70 | 29.50 | 18,895 | Illumina Hiseq X Ten, Nanopore, and Hi- C | Gao et al. (2021) |
| | | | 788.40 | 21.80 | | Illumina NextSeq, Nanopore, and Hi-C | Hai et al. (2022) |
| *Ictalurus punctatus* | Channel catfish | 2n = 58 | 783.00 | 7.73 | 26,661 | Illumina GAIIx, HiSeq, and PacBio | Liu et al. (2016) |
| | | | 845.40 | 7.25 | 21,556 | Illumina HiSeq | X. Chen et al. (2016) |
| *Ictalurus furcatus* | Blue catfish | 2n = 58 | 841.86 | 28.20 | 30,971 | Illumina NovaSeq and PacBio | Wang, Su, et al. (2022) |
| *Silurus meridionalis* | Southern catfish | 2n = 58 | 738.90 | 28.04 | 22,965 | Illumina HiSeq X Ten, Nanopore, and Hi- C | Zheng et al. (2021) |
| *Bagarius yarrelli* | Giant devil catfish | 2n = 56 | 571.00 | 3.10 | 19,027 | Illumina HiSeq X Ten and PacBio | Jiang et al. (2019) |
| *Clarias batrachus* | Walking catfish | 2n = 104 | 821.80 | 0.36 | 22,914 | Illumina HiSeq | Li et al. (2018) |

approach is the use of transcriptome analysis. It involves studying the complete RNA transcripts produced by the genome in a specific cell, tissue, or organism at a particular developmental stage or under certain conditions (Chu & Corey, 2012; Liu, 2007; The Aquaculture Genomics, Genetics and Breeding Workshop et al., 2017). RNA sequencing, often known as RNA-Seq, is a method that involves sequencing RNA using next-generation sequencing technology (Chu & Corey, 2012). This molecular analysis is crucial in catfish research since it enables researchers to comprehend and compare expression patterns among different genes and investigate the molecular mechanisms that govern diverse biological activities at a given time and condition. A considerable number of transcriptome studies have been done on several catfish species. A few notable ones in various catfish species are described here.

An accurate and complete transcriptome assembly was produced using deep sequencing on several tissues of channel catfish (*I. punctatus*) (Jin et al., 2016; Liu et al., 2016). Based on a high-quality reference genome sequence for *I. punctatus* (Table 1), Liu et al. (2016) successfully assembled transcriptomes from the skin of scaled and scaleless *I. punctatus* catfish to examine differentially expressed genes during scale regeneration. The genomic and transcriptomic data during scale regeneration permitted comprehensive analysis of the genomic basis of the most apparent physical trait of catfish — the evolutionary loss of scales. They found that the loss observed in scaleless is due to a deficiency in secretory calcium-binding phosphoproteins (SCPP) genes. According to Wang, Montague, et al. (2022), transcriptome analysis reveals that the blue catfish, *I. furcatus* sperm, undergo significant changes in gene expression during cryopreservation. Their RNA-sequencing analysis revealed 849 genes that were upregulated following cryopreservation. These genes include members from all five mitochondrial electron transport chain complexes, indicating an enhancement in oxidative phosphorylation processes. This results in the excessive generation of reactive oxygen species (ROS), which are linked to cell death (Wang, Montague, et al., 2022).

Transcriptome research is also frequently carried out to elucidate the gene expression patterns under various conditions, such as disease resistance. For example, Ruan et al. (2022) identified the pivotal genes in immune response. It elucidated the gene regulatory network of *P. hypophthalmus* when infected with *Aeromonas hydrophila*, an emerging bacterial disease in aquaculture. Their findings show the impact of *A. hydrophila* infection on *P. hypophthalmus* at the transcriptome level, specifically at 0, 4, 12, 24, 36, and 48 hr. It allowed the identification of differentially expressed genes (DEGs) between each major infection period (4, 12, and 24 hr). The infection of *P. hypophthalmus* influences the expression of genes involved in the immune system, influencing numerous biological processes (Ruan et al., 2022). Understanding disease pathogens aids in the development of more

effective medicines, vaccinations, and management techniques to combat this specific disease (Lu & Luo, 2020; Yue & Wang, 2017). With such information, the vast body of research on transcriptome sequencing in catfish production will increase our understanding of the species, refine our methods, and ensure the long-term viability of this aquaculture industry.

## Linkage Mapping, Quantitative Trait Loci (QTL), and Marker-Assisted Selection (MAS)

Traditional selective breeding procedures such as selection, crossbreeding, and hybridisation remain the major genetic approaches to improve aquaculture production. However, advances in genomics have led to more advanced techniques of QTL and MAS. QTL mapping, in particular for MAS, was initiated for effective and accurate selection at the turn of the century (Liu, 2003; Liu & Cordes, 2004; Ozaki et al., 2017; Yue, 2014). A linkage map is an ordered sequence of genetic markers positioned along the whole of the chromosomes in the genome. The construction of a linkage map involves four key components: polymorphic markers, genotyping tools, reference families, and software for analysing the linkage between pairwise markers and among markers (Liu & Cordes, 2004; Ozaki et al., 2017; Yue, 2014). QTL and MAS are important concepts in genetics and breeding.

Research in aquaculture improvement has mostly focused on the more economically important traits, including disease resistance, growth rate, fillet yield, and tolerance to environmental stressors like temperature fluctuations or low oxygen levels (Yue, 2014). QTLs have been identified and mapped in a number of aquaculture species: tilapia (*Oreochromis* spp.), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), yellowtail amberjack, *Seriola quinqueradiata*, Japanese flounder (*Paralichthys olivaceus*), giant tiger prawn (*Penaeus monodon*), kuruma prawn (*Penaeus japonicus*), Pacific white shrimp, *Litopenaeus vannamei*, as well as Pacific oyster, *Crassostrea gigas* (Lu & Luo, 2020; Ozaki et al., 2017; The Aquaculture Genomics, Genetics and Breeding Workshop et al., 2017; Yue & Wang, 2017; Yue, 2014). Researchers have used QTL mapping techniques to identify regions of the catfish genome associated with these traits, such as head size, weight, hypoxia tolerance, heat stress or disease resistance that are important to breeders for genetic improvement (Liu & Cordes, 2004; Yue, 2014). However, in catfish, previous studies had mainly focused on the Channel catfish, *I. punctatus* and blue catfish, *I. furcatus* (Jin et al., 2016; Liu, 2003; The Aquaculture Genomics, Genetics and Breeding Workshop et al., 2017).

To implement MAS, DNA is tightly linked to QTL for the traits of interest that need to be identified, as determined by QTL mapping or association studies (Ozaki et al., 2017; Yue, 2014). By identifying these markers, breeders can more efficiently select for desired traits in breeding populations. Traits with low inheritance, late in-development expression, or difficulty

in measuring are ideal candidates for improvement through MAS (Yue, 2014). These findings of traits offer a solid foundation for exploring QTL within breeding populations. Though MAS has been applied in fish aquaculture breeding programmes (Ozaki et al., 2017), there has been little investigation on QTL and MAS in pangasiid catfishes up to this point.

## Phylogenetics, Population Genetics, and Mitogenomics

Since the late 1990s, a multitude of phylogenetic analyses of the Pangasiidae family have been conducted. The first molecular phylogenetic study among pangasiid catfish species was conducted by Pouyaud et al. (1998) using 23 enzyme loci on 18 genera of *Pangasius* and *Helicophagus* species. Their study followed a systematic revision of the Asian catfish family Pangasiidae (Roberts & Vidthayanon, 1991) based on morphological characters of specimen types and other materials of pangasiid fishes on 39 nominal species/subspecies. Their study effectively addressed the systematics and phylogenetic relationships of this group. Pouyaud et al. (2000) further expanded their phylogenetic studies on the family Pangasiidae based on combined allozyme data and partial mitochondrial cytochrome *b* gene sequences, with two additional species (20 species). Three monophyletic groups were observed: Group 1 was composed of *P. hypophthalmus* and *P. gigas*, Group 2 was composed of *H. waandersii* and *Helicophagus typus*, and Group 3 was composed of *P. lithostoma*, *Pangasius nieuwenhuisii*, *P. humeralis*, *P. kinabatanganensis*, *Pangasius* sp. nov. 1 (*Pangasius kunyit*), *P. krempfi*, *P. larnaudii*, *P. conchophilus*, *P. nasutus*, *Pangasius djambal*, *P. bocourti*, *Pangasius* sp. nov. *2*, *P. sanitwongsei*, *Pangasius pangasius*, *P. macronema*, and *P. polyuranodon*. However, phylogenetic relationships within Group 3 were not resolved at the internal nodes (Pouyaud et al., 2000). Although a few pairs of closely related species could be demonstrated, the poor resolution of most internal nodes impeded the full resolution of the phylogeny. Their results were congruent with the previous taxonomic hypothesis of the genus *Pangasius* (Pouyaud et al., 1998; Roberts & Vidthayanon, 1991).

Pouyaud et al. (2004) re-analysed the phylogenetic relationships within the family Pangasiidae using mitochondrial 12S rDNA gene sequences to re-affirm their earlier findings. Four genera, i.e., *Pteropangasius* (accepted name: *Pseudolais*), *Helicophagus*, *Pangasianodon*, and *Pangasius*, were identified using a molecular clock or evolutionary calibration based on two mitochondrial DNA genes (12s rDNA and cytochrome *b*). *Pangasius* was found to be the most derived genus. The analysis hypothesised a divergence time of 7-11 mya for the four pangasiid genera and a second episode of speciation characterised by explosive radiation around 5-8 mya for the genus *Pangasius* (Pouyaud et al., 2004). Phylogenetic reconstructions of the family Pangasiidae based on 16S rRNA and *cytochrome oxidase subunit 1* (COI) genes could not fully resolve the relationships of several species in the family (Baharuddin,

2016). However, they were sufficiently efficient for the resolution of the investigated species into four pangasiid genera. A recent mitogenomic study by Abdul Halim et al. (2023) revealed that *P. nasutus* and *P. conchophilus* are sister species with high support (BP: 100%). However, the genetic distance between both was very low at 0.6%, which calls for further investigation. It highlights the contentious phylogenetics and evolutionary relationships of the family Pangasiidae, as genetic distances between several species pairs are comparable to conspecific levels (Duong, Nguyen, et al., 2023; Sriphairoj et al., 2018). The efficacy of COI-based DNA barcoding in discriminating between native and invasive species from the family Pangasiidae in the Pahang River, Malaysia, has also been documented (Baharuddin et al., 2023).

The selection of appropriate and effective molecular markers based on function (protein-coding vs non-coding vs structural RNA) or genomic type (e.g., mitochondrial vs nuclear) is essential for achieving research goals such as a precise phylogenetic reconstruction and species identification (Jamaluddin, 2017). Incorporating mitochondrial and nuclear DNA allows researchers to investigate the impact of incomplete lineage sorting or gene introgression on species delineation in greater depth (Jamaluddin, 2017). Because of their higher mutation rates, mitochondrial DNA genes are more effective in resolving terminal taxa, whereas nuclear DNA genes are well conserved, allowing them to provide deeper taxonomic indications (Miya et al., 2003; Satoh et al., 2016). Karinthanyakit and Jondeung (2012) combined complete mitochondrial cytochrome b (*cyt b*), 12S rRNA, tRNA-Val, and 16S rRNA and partial nuclear recombination-activating gene 1 (*rag1*) to reconstruct the molecular phylogenies of Thai pangasiid and schilbid catfishes. Four significant clades were discovered within the family Pangasiidae, synonymous with the four genera based on mitochondrial cytochrome *b*: *Pangasius*, *Pseudolais*, *Helicophagus*, and *Pangasianodon* (Karinthanyakit & Jondeung, 2012). However, the concatenated analyses showed a marked improvement over the single-locus studies in terms of resolution, the number of well-supported nodes, and the accuracy with which the phylogeny of closely related species was estimated.

The first genetic data applying microsatellite markers in pangasiid was reported by Volckaert et al. (1998). Six loci demonstrated allelic variation in three species of catfishes, i.e., *Clarias gariepinus*, *C. batrachus*, and *P. hypophthalmus*. However, the study only revealed the preliminary genetic structure of wild and farmed populations of the three species. Development of microsatellite markers requires a high initial cost and is time-consuming. The various steps in primer design, testing, and optimising polymerase chain reaction (PCR) conditions are tedious and labour-intensive (Liu, 2007; Liu & Cordes, 2004). Since the pioneering study of Volckaert et al. (1998), developing novel primers and their utilisation have rapidly increased, especially in population genetic studies. Z. S. Hogan and May (2002)

designed 27 primer pairs of microsatellite loci which were tested on five species of pangasiid catfishes (*P. krempfi*, *P. bocourti*, *P. conchophilus*, *P. pleurotaenia*, and *H. waandersii*) from the Mekong River Basin. All 27 loci were polymorphic in at least one species, while 15 were polymorphic in at least three. In another study, 11 microsatellite DNA markers for the critically endangered Mekong giant catfish, *P. gigas*, were designed from captive fishes. These microsatellites were also tested on four closely related species (*P. bocourti*, *P. conchophilus*, *P. larnaudii*, and *P. sanitwongsei*) which successfully cross-amplified, demonstrating the usefulness of these markers for assessing genetic diversity in *P. gigas* and other closely related *Pangasius* species (Ngamsiri et al., 2007). In addition to their extensive usage in population genetics, microsatellite markers have also been widely employed in aquaculture and fisheries research, including investigations of genome mapping, breeding studies for parentage, kinships, and QTL marker development (Liu, 2007; Liu & Cordes, 2004; Ozaki et al., 2017; Yue, 2014).

The utilisation of the whole mitochondrial genome, also referred to as mitogenome, generates reliable and robust data with its abundance of phylogenetically informative sites (Miya et al., 2003; Sam et al., 2021); in recent years, mitogenomic research has gained much importance. The assembly of whole mitogenomes is becoming indispensable for genome evolution studies and for establishing mtDNA reference databases for biodiversity assessment and monitoring (Miya et al., 2003; Sato et al., 2018; Satoh et al., 2016). The last 10 years have seen an explosion of mitogenome investigations, which have allowed for a deeper understanding of the evolutionary relationships among various ray-finned fish families (class Actinopterygii) (Satoh et al., 2016). Yet, data on the mitogenomes of pangasiid species is insufficiently explored; only a few species of mitogenomes are available for the pangasiids (Table 2).

Nevertheless, there is still limited documentation on the region's population structure and genetic variation of pangasiid catfishes. To manage productive fisheries, conserve genetic resources and harvest populations of these catfishes sustainably; it is crucial to understand the genetic diversity and structure of the populations. This information is crucial as knowledge of the precise identification and genetic variability is important in selecting genetically rich broodstocks compatible with a systematic aquaculture programme. Moreover, any restocking programmes require the donor and host populations to be genetically compatible and of the same species (through DNA barcoding). Thus, studying the genetic diversity within catfish populations is essential for maintaining and conserving wild and capture populations. Moreover, information on the phylogenetic relationships of the pangasiid group would be very useful in selecting closely related species as potential candidates for a hybridisation programme.

Table 2
*Available mitogenomes of pangasiid catfishes*

| Species | Sizes (bp) | Accession no. | References |
|---|---|---|---|
| *Pangasius bocourti* | 16,522 | MN842723 | J. Chen et al. (2020) |
| *Pangasius conchophilus* | 16,470 | OQ078744-OQ078745 | Abdul Halim et al. (2023) |
| *Pangasius krempfi* | 16,475 | MZ272453 | Duong, Pham, et al. (2023) |
| *Pangasius larnaudii* | 16,471 | AP012018 | Zhang et al. (2021) |
| *Pangasius mekongensis* | 16,462 | MZ272451 | Duong, Pham, et al. (2023) |
| *Pangasius nasutus* | 16,465 | OP901624, OQ078746, OQ078747 | Abdul Halim et al. (2023) |
| *Pangasius pangasius* | 16,472 | KX950698 | Unpublished |
| *Pangasius sanitwongsei* | 16,536 | MN809630 | Wei et al. (2020) |
| *Pangasianodon gigas* | 16,533 | AY762971 | Jondeung et al. (2007) |
| *Pangasianodon hypophthalmus* | 16,522 | KC846907 | Zhao et al. (2014) |

## AQUACULTURE AND ECONOMIC SIGNIFICANCE OF THE PANGASIIDS

Aquaculture is the fastest-growing food-production industry in agriculture, with significant potential for increased supply to meet human protein demands. The major cultured species in the family Pangasiidae (catfishes) are *P. hypophthalmus* (striped catfish) (Cacot, 1998; Griffiths et al., n.d.; Legendre, 1998), *P. gigas* (Mekong giant catfish) (Legendre, 1998; Sriphairoj et al., 2007), *P. bocourti* (Basa catfish) (Cacot, 1998; Legendre, 1998), and *P. djambal* (Jambal catfish) (Iswanto & Tahapari, 2014; Legendre et al., 2000). Pangasiid catfishes have various advantageous traits that make them good candidates for aquaculture. They have rapid growth rates, are well adapted to poor water quality habitats and are omnivorous (Cacot, 1998; Griffiths et al., n.d.; Legendre, 1998).

Vietnam is the world's biggest producer of *Pangasius*. Its production was estimated to be two billion USD a year, accounting for 70 to 80% of global *Pangasius* production, which is exported to more than 126 countries (Gisbert et al., 2022; Griffiths et al., n.d.; Phuong & Oanh, 2010). Vietnam's Mekong Delta is home to centuries of river catfish culture, making it a valuable resource for aquaculture operations (Cacot, 1998; Gisbert et al., 2022; Phuong & Oanh, 2010). In the early 1960s, *P. bocourti* and *P. hypophthalmus*, locally referred to as Basa and Tra fish, were farmed in small cages and ponds, respectively (Cacot, 1998; Griffiths et al., n.d.; Phuong & Oanh, 2010). Both originate from the Mekong River and Chao Phraya River, are fast-growing, omnivorous, air-breathing species, adaptable to low dissolved oxygen levels (Cacot, 1998; Gisbert et al., 2022; Griffiths et al., n.d.; Legendre, 1998), making them highly amenable to aquaculture. In addition, juvenile *P. hypophthalmus*, commonly referred to as "iridescent sharks", is very

popular in the pet trade. Their black bodies exhibit a blue iridescence and a generalised shark form when young (Armbruster, 2011).

*Pangasianodon hypophthalmus* was introduced into Malaysia, Indonesia, the Philippines, China, Taiwan, Guam, Bangladesh, and India for aquaculture (Griffiths et al., n.d.). It has since been successfully established as a successful feral species in the introduced waters. For instance, it was first brought into Malaysia from Thailand in the 1980s and was successfully produced in captivity by induced spawning, overcoming supply limitations of fingerlings (Cacot, 1998; Griffiths et al., n.d.; Mohamed Yusoff et al., 2019). Currently, the pangasiid captive catfish are cultured in floating cages along the Pahang River, from Kuala Tembeling to Kuala Pahang. Although the Pahang River is home to three indigenous species: *H. waandersii* (Patin Muncung), *P. micronemus* (Patin Lawang/Juara), and *P. nasutus* (Patin Buah), the introduced species, *Pangasianodon hypothalamus*, popularly known as 'Patin Sangkar', is Malaysia's most widely cultivated catfish species (Baharuddin, 2016; Hassan et al., 2011; Mohamed Yusoff et al., 2019). In addition to the major cultured pangasiid species mentioned above, other potential aquaculture candidates are *P. nasutus* and *P. krempfi*.

### *Pangasius nasutus:* A Rising Aquaculture Species

In recent years, there has been growing interest in the culture of the native 'Patin Buah', *P. nasutus*, in Peninsular Malaysia and Indonesia. It is a well-known delicacy, but unfortunately, at a market price of > USD 35/kg, it is beyond the budget of most locals (personal communication with local anglers). Furthermore, as the supplies are based on wild capture, these natural resources would be depleted if not strategically managed (Chong et al., 2010; Hassan et al., 2011). A well-structured programme modelled along the lines of the "Catfish Asia" project (Legendre, 1998; Legendre et al., 2000) should be conducted should be followed in other Southeast Asian countries. A culture programme of *P. nasutus* has already been successfully initiated by the Freshwater Fisheries Research Division (FRI Glami-Lemi), Malaysia, through a breeding development programme (Jaapar, 2020) from wild broodstock. The main objective of this facility was to restock the Pahang River as well as several other major rivers in the state of Pahang, which was known to support good numbers before the decline in the last decade (Jaapar, 2020; Jaapar et al., 2021).

A novel fish induction breeding technique was recently introduced in *P. nasutus* (Jaapar, 2020). The procedure involved stimulating mature fishes to reproduce in captivity by introducing pituitary hormones and releasing eggs and sperm from ripe gonads as a result of the stimulation (Jaapar, 2020). Using hormones in the induced breeding of *P. nasutus* enables farmers to produce large quantities of seeds. There are various advantages of using induced breeding to produce seeds:

(1) creates an efficient breeding programme for seed production, (2) allows non-seasonal breeding, (3) produces numerous seeds per induction, (4) allows for the selection of higher-quality parent fish, (5) managing the survival of seeds, and (6) guarantees a lucrative return (Jaapar, 2020). In terms of technical aspects, tagging has also been found to be useful in supporting broodstock management (Jaapar, 2020). The tagging prevents repeated injections of the same fish and inbreeding. This preliminary success could support the freshwater 'Patin Buah', *P. nasutus* industry.

The 'Patin Buah' has exceptional potential for the food fish export industry with its white-fine-grained and sweet flesh, compared to the yellowish flesh of *P. hypophthalmus* (Hassan et al., 2011; Tahapari et al., 2011). The same characteristics have led to increasing interest in *P. bocourti* (Vietnam) and *P. djambal* (Indonesia) (Iswanto & Tahapari, 2014). Both species are extensively farmed to produce more white-meat catfish in their regions. Hybridisation between *P. nasutus* and other closely related catfish is also being trialled. It is hoped that this would produce hybrids with combined qualities of superior meat and faster growth than the parents (Hassan et al., 2011; Iswanto & Tahapari, 2014; Payseur & Rieseberg, 2016).

### *Pangasius krempfi*: Another Rising Aquaculture Species in Coastal Areas

Unlike most pangasiids, which live in freshwater, *P. krempfi* spends part of its life in brackish and coastal areas along the Mekong Delta, Vietnam (Duong, Nguyen, et al., 2023; Z. Hogan et al., 2007). Because of its high meat quality and commercial value, it is considered the most expensive catfish (Vu et al., 2020). This species has gained increased interest in aquaculture in the Mekong Delta, Vietnam. However, the domestication process, which started in 2006 (Trinh et al., 2005), has not been fully established. One of the obstacles is the reproductive migratory behaviour of the species, which hinders obtaining a high number of broodstock (Roberts & Baird, 1995; Vu et al., 2020; Z. Hogan et al., 2007). Farmers in the Mekong coastal areas still rely on wild fingerlings for pond culture with some profits (personal communication by farmers in the delta). Admittedly, this is not sustainable, so greater efforts are needed to conserve the species.

### Hybridisation and Gene Introgression Among Pangasiid Catfishes

The last few decades have seen increasing research into the evolutionary processes of hybridisation and introgression (the transfer of genes across species-mediated mostly by backcrossing in aquaculture species (Payseur & Rieseberg, 2016; Sriphairoj et al., 2018). Hybridisation is typically employed for genetic improvement by crossbreeding species to outperform either of their parents or combine beneficial traits from both parents.

Interspecific hybridisation between pangasiid species, such as *P. hypophthalmus* X *P. gigas*, *P. hypophthalmus* X *P. bocourti*, and *P. hypophthalmus* X *P. larnaudii*, has

been well established mainly in Thailand for their superior characteristics in growth, survival rate, and meat quality (Gisbert et al., 2022; Sriphairoj et al., 2018). In addition, hybridisation between *P. hypophthalmus* X *P. nasutus* has also been trialled (Hassan et al., 2011; Iswanto & Tahapari, 2011, 2014; Mohamed Yusoff et al., 2019; Samad et al., 2020; Tahapari et al., 2011). However, Sriphairoj et al. (2018) raised concerns about the genetic introgression of these farmed animals into wild populations as interspecific hybridisation among pangasiid species has been known to produce reproductively viable hybrids, such as in the *P. hypophthalmus* X *P. gigas* hybrid. It will be detrimental to the ecological health of the original parents in the habitat when hybrids are superior.

## FUTURE PERSPECTIVES

Genomics will likely play a pivotal role in the conservation, breeding, and improvement of pangasiid catfish species. This review briefly describes the current trends in aquaculture, from traditional molecular genetics to NGS genomics approaches in the conservation efforts of the family Pangasiidae catfishes in Southeast Asia. With the rapid advancements in NGS technology and genomic analysis, aquaculture and fisheries management will see a paradigm shift. The following provides an insight into the potential future.

### Genomic Sequencing and Annotation

Recent developments in sequencing technology have the potential to greatly improve the speed and cost of whole-genome sequencing, opening the door to the rapid collection of genomic data for a wide range of catfish species. Furthermore, identifying genes and their roles through genome annotation efforts will yield important insights into features associated with growth, tolerance to illness, and environmental adaptation.

### Conservation Efforts

Genomic research permits a comprehensive understanding of the genetic variability, population structure, and adaptation of pangasiid catfish species across numerous environments. This information will greatly aid in identifying and protecting genetically distinct populations and developing successful conservation methods, making it vital for conservation efforts.

### Breeding and Improvement

Selective breeding programmes will benefit from using genomic technologies, making it possible to identify desired traits, including disease resistance, growth rate, and stressor tolerance. It will initiate the development of improved strains of pangasiid catfish with higher productivity and resilience. Understanding the genetic basis of disease resistance will enable the development of pangasiid catfish breeds that are more resistant to prevalent diseases, reducing the need for antibiotics and improving overall fish health, as genomic research may focus on identifying genes related to disease resistance in pangasiid catfishes. The beneficial outcome is that the economic losses brought on by diseases in aquaculture

could be effectively addressed. In addition, with the increasing use of MAS and genomic selection approaches, the aquaculture industry will be able to selectively increase certain traits in pangasiid catfish populations.

### Market Competition and Demand

It is essential to meet customer demands for high-quality and environmentally friendly seafood. Utilising genomics in catfish aquaculture can enhance farmers' competitiveness by providing higher-quality products.

## CONCLUSION

The convergence of aquaculture and genomics in catfish farming has already led to significant advancements in breeding, disease resistance, and overall production efficiency. Continued research and application of genomic knowledge are expected to further revolutionise the catfish industry, making it more sustainable, efficient, and responsive to market demands. Genomic research on pangasiid catfishes is still in its infancy, and currently, only basic data is available on the species investigated, and very few in-depth functional studies have been conducted. The limited fundamental data impedes exhaustive genomic applications in the family Pangasiidae. Nevertheless, the striped catfish (*P. hypophthalmus*) draft genome sequence is already available, and the complete reference genome sequence for the channel catfish (*I. punctatus*) marks an important milestone for structural genomics and functional genomics studies of other species, such as pangasiid catfishes. While this overview of pangasiid catfishes is not exhaustive, the significant issues highlighted in the published studies are covered. Genomics offers enormous potential in the aquaculture sector and, thus, human well-being.

## ACKNOWLEDGEMENTS

This work was supported by the Ministry of Higher Education (Malaysia) under the Long-Term Research Grant Scheme (LRGS/1/2019/UPM/01/1/1) – 'Selective breeding for high quality seed productions and establishment of larval and juvenile culture techniques of *Pangasius nasutus*'.

## REFERENCES

Abdul Halim, S. A. A., Esa, Y., Gan, H. M., Zainudin, A. A., & Mohd Nor, S. A. (2023). The complete mitochondrial genomes of *Pangasius nasutus* and *P. conchophilus* (Siluriformes: Pangasiidae). *Mitochondrial DNA Part B: Resources*, *8*(1), 38–41. https://doi.org/10.1080/23802359.2022.2158694

Armbruster, J. W. (2011). Global catfish biodiversity. In P. H. Michaletz & V. H. Travnichek (Eds.), *Conservation, Ecology, and Management of Catfish: The Second International Symposium* (pp. 15–37). American Fisheries Society. https://doi.org/10.47886/9781934874257.ch3

Baharuddin, H. (2016). *Morphometric, phylogenetic analyses and DNA barcoding of pangasiid catfishes (Teleostei: Pangasiidae) in Peninsular Malaysia* [Doctoral dissertation, Universiti Malaya]. ProQuest Dissertations and Theses Global. https://www.proquest.com/dissertations-theses/morphometric-phylogenetic-analyses-dna-barcoding/docview/2848787122/se-2?accountid=27932

Baharuddin, H., Amatul-Samahah, M. A., Rizman-Idid, M., & Muniandy, S. (2023). DNA barcoding and phylogenetics relationship of pangasiid catfishes in Peninsular Malaysia revealed the impacts of aquaculture on the native species conservation. *Hydrobiology*, *2*(2), 431–445. https://doi.org/10.3390/hydrobiology2020028

Cacot, P. (1998). *Description of the sexual cycle related to the environment and set up of the artificial propagation in* Pangasius bocourti *(Sauvage, 1880) and* Pangasius hypophthalmus *(Sauvage, 1878), reared in floating cages and in ponds in the Mekong Delta*. https://horizon.documentation.ird.fr/exl-doc/pleins_textes/doc34-08/010020339.pdf

Chen, J., Gao, T., Chen, M., & Ou, Q. (2020). Next-generation sequencing of the mitochondrial genome of *Pangasius bocourti* (Siluroidei: Pangasiidae). *Mitochondrial DNA Part B: Resources*, *5*(2), 1779–1780. https://doi.org/10.1080/23802359.2020.1749148

Chen, X., Zhong, L., Bian, C., Xu, P., Qiu, Y., You, X., Zhang, S., Huang, Y., Li, J., Wang, M., Qin, Q., Zhu, X., Peng, C., Wong, A., Zhu, Z., Wang, M., Gu, R., Xu, J., Shi, Q., & Bian, W. (2016). High-quality genome assembly of channel catfish, *Ictalurus punctatus*. *GigaScience*, *5*(1), s13742-016-0142-5. https://doi.org/10.1186/s13742-016-0142-5

Chong, V. C., Lee, P. K. Y., & Lau, C. M. (2010). Diversity, extinction risk and conservation of Malaysian fishes. *Journal of Fish Biology*, *76*(9), 2009–2066. https://doi.org/10.1111/j.1095-8649.2010.02685.x

Chu, Y., & Corey, D. R. (2012). RNA sequencing: Platform selection, experimental design, and data interpretation. *Nucleic Acid Therapeutics*, *22*(4), 271–274. https://doi.org/10.1089/nat.2012.0367

Duong, T. Y., Pham, L. T. K., Le, X. T. K., Nguyen, N. T. T., Nor, A. M., & Le, T. H. (2023). Mitophylogeny of pangasiid catfishes and its taxonomic implications for Pangasiidae and suborder Silurodei. *Zoological Studies*, *62*, e48. https://doi.org/10.6620%2FZS.2023.62-48

Duong, T.-Y., Nguyen, N.-T. T., Tran, D. D., Le, T. H., & Nor, S. A. M. (2023). Multiple genetic lineages of anadromous migratory Mekong catfish *Pangasius krempfi* revealed by mtDNA control region and cytochrome *b*. *Ecology and Evolution*, *13*(2), e9845. https://doi.org/10.1002/ece3.9845

Fricke, R., Eschmeyer, W. N., & Van der Laan, R. (Eds.) (2024). *Eschmeyer's catalog of fishes: Genera, species, references*. https://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp

Froese, R., & Pauly, D. (Eds.) (2024). *FishBase* (version 06/2024). www.fishbase.org

Gao, Z., You, X., Zhang, X., Chen, J., Xu, T., Huang, Y., Lin, X., Xu, J., Bian, C., & Shi, Q. (2021). A chromosome-level genome assembly of the striped catfish (*Pangasianodon hypophthalmus*). *Genomics*, *113*(5), 3349–3356. https://doi.org/10.1016/j.ygeno.2021.07.026

Gisbert, E., Luz, R. K., Fernández, I., Pradhan, P. K., Salhi, M., Mozanzadeh, M. T., Kumar, A., Kotzamanis, Y., Castro-Ruiz, D., Bessonart, M., & Darias, M. J. (2022). Development, nutrition, and rearing practices of relevant catfish species (Siluriformes) at early stages. *Reviews in Aquaculture*, *14*, 73–105. https://doi.org/10.1111/raq.12586

Goodwin, S., McPherson, J. D., & McCombie, W. R. (2016). Coming of age: Ten years of next-generation sequencing technologies. *Nature Reviews Genetics*, *17*, 333–351. https://doi.org/10.1038/nrg.2016.49

Griffiths, D., Khanh, P. V., & Trong, T. Q. (n.d.). Pangasius hypophthalmus *(Sauvage, 1878) [Pangasiidae]*. Food and Agriculture Organization of the United Nations. https://openknowledge.fao.org/server/api/

core/bitstreams/40ca4f4a-fc6d-4947-974a-dc86d1ed71bc/content

Gustiano, R. (2009). Pangasiid catfishes of Indonesia. *Buletin Plasma Nutfah*, *15*(2), 91–100.

Gustiano, R., & Pouyaud, L. (2005). Riverine catfishes of Kalimantan, Pangasiidae: Diagnosis, distribution, and ecology. *Indonesian Fisheries Research Journal*, *11*(2), 59–66. https://doi.org/10.15578/ifrj.11.2.2005.59-66

Hai, D. M., Yen, D. T., Liem, P. T., Tam, B. M., Huong, D. T. T., Hang, B. T. B., Hieu, D. Q., Garigliany, M. M., Coppieters, W., Kestemont, P., Phuong, N. T., & Farnir, F. (2022). A high-quality genome assembly of striped catfish (*Pangasianodon hypophthalmus*) based on highly accurate long-read HiFi sequencing data. *Genes*, *13*(5), 923. https://doi.org/10.3390/genes13050923

Hassan, A., Ambak, M. A., & Samad, A. P. A. (2011). Crossbreeding of *Pangasianodon hypophthalmus* (Sauvage, 1878) and *Pangasius nasutus* (Bleeker, 1863) and their larval development. *Journal of Sustainability Science and Management*, *6*(1), 28–35.

Hogan, Z. S., & May, B. P. (2002). Twenty-seven new microsatellites for the migratory Asian catfish family Pangasiidae. *Molecular Ecology Resources*, *2*(1), 38–41. https://doi.org/10.1046/j.1471-8286.2002.00139.x

Hogan, Z., Baird, I. G., Radtke, R., & Vander Zanden, M. J. (2007). Long distance migration and marine habitation in the tropical Asian catfish, *Pangasius krempfi*. *Journal of Fish Biology*, *71*(3), 818–832. https://doi.org/10.1111/j.1095-8649.2007.01549.x

Inger, R. F., & Chin, P. K. (1962). *The fresh-water fishes of North Borneo* (Vol. 45). Chicago Natural History Museum.

International Union for Conservation of Nature. (2023). *The IUCN Red List of Threatened Species*. Version 2022-2. https://www.iucnredlist.org

Iswanto, B., & Tahapari, E. (2011). Embriogenesis dan perkembangan larva patin hasil hibridisasi antara betina ikan patin siam (*Pangasianodon hypophthalmus* Sauvage, 1878) dengan jantan ikan patin jambal (*Pangasius djambal* Bleeker, 1846) dan jantan patin nasutus (*Pangasius nasutus* Bleeker, 1863) [Embryogenesis and development of catfish larvae resulting from hybridization between female Siamese catfish (*Pangasianodon hypophthalmus* Sauvage, 1878) with male jambal catfish (*Pangasius djambal* Bleeker, 1846) and male nasutus catfish (*Pangasius nasutus* Bleeker, 1863)]. *Jurnal Riset Akuakultur*, *6*(2), 169-186. https://doi.org/10.15578/jra.6.2.2011.169-186

Iswanto, B., & Tahapari, E. (2014). Keragaan pemijahan buatan antara ikan patin siam (*Pangasianodon hypophthalmus*) betina dan ikan patin jambal (*Pangasius djambal*) jantan dan ikan patin nasutus (*Pangasius nasutus*) jantan [Artificial spawning performance between female Siamese catfish (*Pangasianodon hypophthalmus*) and male jambal catfish (*Pangasius djambal*) and male nasutus catfish (*Pangasius nasutus*)]. *Jurnal Riset Akuakultur*, *9*(2), 191–201. https://doi.org/10.15578/jra.9.2.2014.191-201

Jaapar, M. Z. (2020). *Manual pembenihan Patin Buah* [Patin Buah seeding manual]. Fisheries Research Institute.

Jaapar, M. Z., Yusof, M. F., Yusof, H. M., Ramli, N. S. F., Mohamad, S. N., & Jamari, Z. (2021). Effect of different salinity concentrations on hatching rate and larval development of Patin Buah, *Pangasius nasutus* (Bleeker, 1863). *Journal of Applied Aquaculture*, *34*(3), 693-701. https://doi.org/10.1080/10454438.2021.1885556

Jamaluddin, J. A. F. (2017). *Molecular systematics, historical biogeography and population genetics of the Asian spiny eel (Synbranchiformes: Mastacembelidae)* [Doctoral dissertation, Universiti Sains Malaysia]. Repository@USM. https://erepo.usm.my/items/8ca072c8-67ff-4989-a426-2f63857892c4

Jiang, W., Lv, Y., Cheng, L., Yang, K., Bian, C., Wang, X., Li, Y., Pan, X., You, X., Zhang, Y., Yang, J., Li, J., Zhang, X., Liu, S., Sun, C., Yang, J., & Shi, Q. (2019). Whole-genome sequencing of the giant devil catfish, *Bagarius yarrelli*. *Genome Biology and Evolution*, *11*(8), 2071–2077. https://doi.org/10.1093/gbe/evz143

Jin, Y., Liu, S., Yuan, Z., Yang, Y., Tan, S., & Liu, Z. (2016). Catfish genomic studies: Progress and perspectives. In S. MacKenzie & S. Jentoft (Eds.), *Genomics in aquaculture* (pp. 73–104). Academic Press. https://doi.org/10.1016/B978-0-12-801418-9.00004-4

Jondeung, A., Sangthong, P., & Zardoya, R. (2007). The complete mitochondrial DNA sequence of the Mekong giant catfish (*Pangasianodon gigas*), and the phylogenetic relationships among Siluriformes. *Gene*, *387*(1–2), 49–57. https://doi.org/10.1016/j.gene.2006.08.001

Karinthanyakit, W., & Jondeung, A. (2012). Molecular phylogenetic relationships of pangasiid and schilbid catfishes in Thailand. *Journal of Fish Biology*, *80*(7), 2549–2570. https://doi.org/10.1111/j.1095-8649.2012.03303.x

Kim, O. T. P., Nguyen, P. T., Shoguchi, E., Hisata, K., Vo, T. T. B., Inoue, J., Shinzato, C., Le, B. T. N., Nishitsuji, K., Kanda, M., Nguyen, V. H., Nong, H. V., & Satoh, N. (2018). A draft genome of the striped catfish, *Pangasianodon hypophthalmus*, for comparative analysis of genes relevant to development and a resource for aquaculture improvement. *BMC Genomics*, *19*, 733. https://doi.org/10.1186/s12864-018-5079-x

Kottelat, M. (2013). The fishes of the inland waters of Southeast Asia: A catalogue and core bibliography of the fishes known to occur in freshwaters, mangroves and estuaries. *The Raffles Bulletin of Zoology*, *Supplement No. 27*, 1–663.

Legendre, M. (1998). *The Catfish Asia project: Backgrounds, aims and prospects*. https://horizon.documentation.ird.fr/exl-doc/pleins_textes/doc34-08/010020339.pdf

Legendre, M., Pouyaud, L., Slembrouck, J., Gustiano, R., Kristanto, A. H., Subagja, J., Komarudin, O., & Maskur. (2000). *Pangasius djambal*: A new candidate species for fish culture in Indonesia. *Indonesian Agricultural Research and Development Journal*, *22*(1), 1–14.

Li, N., Bao, L., Zhou, T., Yuan, Z., Liu, S., Dunham, R., Li, Y., Wang, K., Xu, X., Jin, Y., Zeng, Q., Gao, S., Fu, Q., Liu, Y., Yang, Y., Li, Q., Meyer, A., Gao, D., & Liu, Z. (2018). Genome sequence of walking catfish (*Clarias batrachus*) provides insights into terrestrial adaptation. *BMC Genomics*, *19*, 952. https://doi.org/10.1186/s12864-018-5355-9

Liu, Z. (2003). A review of catfish genomics: Progress and perspectives. *Comparative and Functional Genomics*, *4*, 259–265. https://doi.org/10.1002/cfg.265

Liu, Z. (Ed.) (2007). *Aquaculture genome technologies*. Blackwell Publishing Ltd. https://doi.org/10.1002/9780470277560

Liu, Z. J., & Cordes, J. F. (2004). DNA marker technologies and their applications in aquaculture genetics. *Aquaculture*, *238*(1–4), 1–37. https://doi.org/10.1016/j.aquaculture.2004.05.027

Liu, Z., Liu, S., Yao, J., Bao, L., Zhang, J., Li, Y., Jiang, C., Sun, L., Wang, R., Zhang, Y., Zhou, T., Zeng, Q., Fu, Q., Gao, S., Li, N., Koren, S., Jiang, Y., Zimin, A., Xu, P., … Waldbieser, G. C. (2016). The channel catfish genome sequence provides insights into the evolution of scale formation in teleosts. *Nature Communications*, *7*, 11757. https://doi.org/10.1038/ncomms11757

Lu, G., & Luo, M. (2020). Genomes of major fishes in world fisheries and aquaculture: Status, application and perspective. *Aquaculture and Fisheries*, *5*(4), 163–173. https://doi.org/10.1016/j.aaf.2020.05.004

Miya, M., Takeshima, H., Endo, H., Ishiguro, N. B., Inoue, J. G., Mukai, T., Satoh, T. P., Yamaguchi, M., Kawaguchi, A., Mabuchi, K., Shirai, S. M., & Nishida, M. (2003). Major patterns of higher teleostean phylogenies: A new perspective based on 100 complete mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, *26*(1), 121–138. https://doi.org/10.1016/S1055-7903(02)00332-9

Mohamed Yusoff, S. F., Christianus, A., Ismail, M. F. S., Esa, Y., Hassan, M. D., Hamid, N. H., Siti Nadia, B. A., & Zulkifle, M. S. (2019). Discrimination analysis of hybrid *Pangasianodon hypophthalmus* (Sauvage, 1983) (♀) × *Pangasius nasutus* (♂) (Bleeker, 1976) and its parental species. *Journal of Survey in Fisheries Sciences*, *5*(2), 49–63.

Moyle, P. B., & Leidy, R. A. (1992). Loss of biodiversity in aquatic ecosystems: Evidence from fish faunas. In P. L. Fiedler & S. K. Jain (Eds.), *Conservation biology* (pp. 127–169). Springer. https://doi.org/10.1007/978-1-4684-6426-9_6

Ngamsiri, T., Nakajima, M., Sukmanomon, S., Sukumasavin, N., Kamonrat, W., Na-Nakorn, U., & Taniguchi, N. (2007). Genetic diversity of wild Mekong giant catfish *Pangasianodon gigas* collected from Thailand and Cambodia. *Fisheries Science*, *73*, 792–799. https://doi.org/10.1111/j.1444-2906.2007.01398.x

Ozaki, A., Araki, K., Aoki, J.-Y., Shimada, Y., Okamoto, H., Usuki, H., Okuzawa, K., Yoshida, K., & Noda, T. (2017). Paradigm shift in fish breeding: Marker-assisted selection to genomic selection. In K. Gruenthal, C. Otoshi, P. Olin, & M. Rust (Eds.), *Genetics and Breeding in Aquaculture: Proceedings of the 44th U.S. – Japan Aquaculture Panel Symposium* (pp. 20-24). U.S. Department of Commerce, National Oceanic and Atmospheric Administration, and National Marine Fisheries Service. https://spo.nmfs.noaa.gov/sites/default/files/TMSPO175_0.pdf

Parenti, L. R. (1996). Phylogenetic systematics and biogeography of phallostethid fishes (Atherinomorpha, Phallostethidae) of Northwestern Borneo, with description of a new species. *Copeia*, *3*, 703–712. https://doi.org/10.2307/1447535

Parenti, L. R., & Lim, K. K. P. (2005). Fishes of the Rajang Basin, Sarawak, Malaysia. *The Raffles Bulletin of Zoology*, *Supplement No. 13*, 175–208.

Payseur, B. A., & Rieseberg, L. H. (2016). A genomic perspective on hybridization and speciation. *Molecular Ecology, 25*(11), 2337–2360. https://doi.org/10.1111/mec.13557

Phuong, N. T., & Oanh, D. T. H. (2010). Striped catfish aquaculture in Vietnam: A decade of unprecedented development. In S. S. de Silva & F. B. Davy (Eds.), *Success stories in Asian aquaculture* (pp. 131–147). Springer. https://doi.org/10.1007/978-90-481-3087-0_7

Pouyaud, L., Gustiano, R., & Legendre, M. (1998). *Phylogenetic relationships among pangasiid catfish species (Siluriformes, Pangasiidae) and new insights on their zoogeography*. https://horizon.documentation.ird.fr/exl-doc/pleins_textes/doc34-08/010020339.pdf

Pouyaud, L., Gustiano, R., & Teugels, G. G. (2004). Contribution to the phylogeny of the Pangasiidae based on mitochondrial 12s rDNA. *Indonesian Journal of Agricultural Science*, *5*(2), 45–62.

Pouyaud, L., Teugels, G. G., Gustiano, R., & Legendre, M. (2000). Contribution to the phylogeny of pangasiid catfishes based on allozymes and mitochondrial DNA. *Journal of Fish Biology*, *56*(6), 1509–1538. https://doi.org/10.1006/jfbi.2000.1279

Roberts, T. R., & Baird, I. G. (1995). Traditional fisheries and fish ecology on the Mekong River at Khone waterfalls in southern Laos. *Natural History Bulletin of the Siam Society*, *43*, 219–262.

Roberts, T. R., & Vidthayanon, C. (1991). Systematic revision of the Asian catfish family Pangasiidae, with biological observations and descriptions of three new species. *Proceedings of the Academy of Natural Sciences of Philadelphia*, *143*, 97–144.

Ruan, Z.-H., Jiang, L.-S., Li, Y.-F., Lu, Z.-Q., Chen, X.-C., Zhang, X., & Liu, W.-S. (2022). Transcriptomic analysis of the immune response against *A. hydrophila* infection in striped catfish *Pangasianodon hypophthalmus*. *Aquaculture*, *547*, 737446. https://doi.org/10.1016/j.aquaculture.2021.737446

Sam, K.-K., Lau, N.-S., Shu-Chien, A. C., Muchlisin, Z. A., & Nugroho, R. A. (2021). Complete mitochondrial genomes of *Paedocypris micromegethes* and *Paedocypris carbunculus* reveal conserved gene order and phylogenetic relationships of miniaturized cyprinids. *Frontiers in Ecology and Evolution*, *9*, 662501. https://doi.org/10.3389/fevo.2021.662501

Samad, A. P. A., Isma, M. F., Humairani, R., & Akmal, Y. (2020). Histological studies on digestive system development and early feeding activity of *Pangasius* hybrid larvae. *Journal of Aquaculture and Fish Health*, *9*(2), 86–94. https://doi.org/10.20473/jafh.v9i2.15810

Sato, Y., Miya, M., Fukunaga, T., Sado, T., & Iwasaki, W. (2018). MitoFish and Mifish pipeline: A mitochondrial genome database of fish with an analysis pipeline for environmental DNA metabarcoding. *Molecular Biology and Evolution*, *35*(6), 1553–1555. https://doi.org/10.1093/molbev/msy074

Satoh, T. P., Miya, M., Mabuchi, K., & Nishida, M. (2016). Structure and variation of the mitochondrial genome of fishes. *BMC Genomics*, *17*, 719. https://doi.org/10.1186/s12864-016-3054-y

Sriphairoj, K., Kamonrat, W., & Na-Nakorn, U. (2007). Genetic aspect in broodstock management of the critically endangered Mekong giant catfish, *Pangasianodon gigas* in Thailand. *Aquaculture*, *264*(1–4), 36–46. https://doi.org/10.1016/j.aquaculture.2006.12.046

Sriphairoj, K., Na-Nakorn, U., & Klinbunga, S. (2018). Species identification of non-hybrid and hybrid pangasiid catfish using polymerase chain reaction-restriction fragment length polymorphism. *Agriculture and Natural Resources*, *52*(1), 99–105. https://doi.org/10.1016/j.anres.2018.05.014

Tahapari, E., Iswanto, B., & Sularto, S. (2011). Keragaan reproduksi ikan patin nasutus (*Pangasius nasutus* Bleeker, 1863) sebagai kandidat ikan budidaya [The reproductive nature of nasutus catfish (*Pangasius nasutus* Bleeker, 1863) as a candidate for aquaculture]. *Jurnal Riset Akuakultur*, *6*(1), 17–30. https://doi.org/10.15578/jra.6.1.2011.17-30

The Aquaculture Genomics, Genetics and Breeding Workshop., Abdelrahman, H., ElHady, M., Alcivar-Warren, A., Allen, S., Al-Tobasei, R., Bao, L., Beck, B., Blackburn, H., Bosworth, B., Buchanan, J., Chappell, J., Daniels, W., Dong, S., Dunham, R., Durland, E., Elaswad, A., Gomez-Chiarri, M., Gosh, K., … Zhou, T. (2017). Aquaculture genomics, genetics and breeding in the United States: Current status, challenges, and priorities for future research. *BMC Genomics*, *18*, 191. https://doi.org/10.1186/s12864-017-3557-1

Trinh, Q. T., Huynh, H. N., Thi, T. V., Nguyen, M. T., & Hoang, Q. B. (2005). *Preliminary results of domestication of Pangasius krempfi*. https://archive.iwlearn.net/mrcmekong.org/download/free_download/proceedings_7TechSym/paper17-Preliminary-results.pdf

Volckaert, F., Hellemans, B., & Pouyaud, L. (1998). *Preliminary data on genetic variation in the genus* Clarias *and Pangasius on the basis of DNA microsatellite loci*. https://horizon.documentation.ird.fr/exl-doc/pleins_textes/doc34-08/010020339.pdf

Vu, A. V., Baumgartner, L. J., Mallen-Cooper, M., Howitt, J. A., Robinson, W. A., So, N., & Cowx, I. G. (2020). Diadromy in a large tropical river, the Mekong: More common than assumed, with greater implications for management. *Journal of Ecohydraulics*, *8*(1), 38–50. https://doi.org/10.1080/24705357.2020.1818642

Wang, H., Montague, H. R., Hess, H. N., Zhang, Y., Aguilar, G. L., Dunham, R. A., Butts, I. A. E., & Wang, X. (2022). Transcriptome analysis reveals key gene expression changes in blue catfish sperm in response to cryopreservation. *International Journal of Molecular Sciences*, *23*(14), 7618. https://doi.org/10.3390/ijms23147618

Wang, H., Su, B., Butts, I. A. E., Dunham, R. A., & Wang, X. (2022). Chromosome-level assembly and annotation of the blue catfish *Ictalurus furcatus*, an aquaculture species for hybrid catfish reproduction, epigenetics, and heterosis studies. *GigaScience*, *11*, giac070. https://doi.org/10.1093/gigascience/giac070

Wei, L., Ye, X., Lv, Y., Teng, Z., Gan, B., Zou, H., Mo, F., & Zhang, S. (2020). Complete mitochondrial genome and phylogenetic position of *Pangasius sanitwongsei* (Siluriformes: Pangasiidae). *Mitochondrial DNA Part B: Resources*, *5*(1), 945–946. https://doi.org/10.1080/23802359.2020.1719915

Yue, G. H. (2014). Recent advances of genome mapping and marker-assisted selection in aquaculture. *Fish and Fisheries*, *15*(3), 376–396. https://doi.org/10.1111/faf.12020

Yue, G. H., & Wang, L. (2017). Current status of genome sequencing and its applications in aquaculture. *Aquaculture*, *468*(Part 1), 337–347. https://doi.org/10.1016/j.aquaculture.2016.10.036

Zhang, K., Liu, Y., Chen, J., Zhang, H., Gong, L., Jiang, L., Liu, L., Lü, Z., & Liu, B. (2021). Characterization of the complete mitochondrial genome of *Macrotocinclus affinis* (Siluriformes; Loricariidae) and phylogenetic studies of Siluriformes. *Molecular Biology Reports*, *48*, 677–689. https://doi.org/10.1007/s11033-020-06120-z

Zhao, H., Kong, X., & Zhou, C. (2014). The mitogenome of *Pangasius sutchi* (Teleostei, Siluriformes: Pangasiidae). *Mitochondrial DNA Part A: The Journal of DNA Mapping, Sequencing, and Analysis*, *25*(5), 342–344. https://doi.org/10.3109/19401736.2013.800492

Zheng, S., Shao, F., Tao, W., Liu, Z., Long, J., Wang, X., Zhang, S., Zhao, Q., Carleton, K. L., Kocher, T. D., Jin, L., Wang, Z., Peng, Z., Wang, D., & Zhang, Y. (2021). Chromosome-level assembly of southern catfish (*Silurus meridionalis*) provides insights into visual adaptation to nocturnal and benthic lifestyles. *Molecular Ecology Resources*, *21*(5), 1575–1592. https://doi.org/10.1111/1755-0998.13338

Pertanika J. Trop. Agric. Sci. 47 (4): 1245 - 1260 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Growth Patterns and Morphometric Characteristics of Female Sakub Sheep Reared by Smallholder Farmers in Brebes Regency of Central Java, Indonesia

**Zaenab Nurul Jannah[1], Panjono[1]*, Sigit Bintara[1], Tri Satya Mastuti Widi[1], Adi Tiya Warman[1], Alek Ibrahim[2], Bayu Andri Atmoko[2], Dayu Lingga Lana[3] and Budi Santosa[3]**

*[1]Department of Animal Production, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*
*[2]Research Center for Animal Husbandry, National Research, and Innovation Agency, Cibinong 16911, Indonesia*
*[3]Animal Husbandry and Veterinary Agency of Brebes Regency, Brebes 52212, Indonesia*

## ABSTRACT

The Indonesian government designated the Sakub sheep as a local breed in 2022, and it is mainly cultivated in Brebes Regency, Central Java. This study investigates the growth patterns and morphometric characteristics of Sakub sheep as a local breed in the Brebes Regency. The data were collected from 195 healthy and non-pregnant female Sakub sheep reared by smallholder farmers. The sheep were categorized into eight age groups, and various morphometric measurements were performed, followed by descriptive data analysis. The results showed that body weight (BW), chest width (CW), and chest depth (CD) experienced optimal growth until 36–48 months with average body weight and size of 47.34 kg, 21.76 cm, and 35.35 cm, respectively. The body size of heart girth (HG), body length (BL), and hip height (HH) grew optimally for 7–12 months at 80.40, 64.73, and 65.08 cm, respectively. Wither height (WH) and hip-width (HW) growth were optimal for 12–24 months at 67.27 and 15.77 cm, respectively, gradually developing into maturity. In conclusion, this study showed the rapid growth of female Sakub sheep, indicating that body weight and size (CW and CD) generally grew to maturity. In contrast, the growth pattern of body size related to BL, WH, HH, and HW showed rapid growth from birth to puberty, followed by a decline in development rate

ARTICLE INFO

*Article history:*
Received: 21 February 2024
Accepted: 15 April 2024
Published: 25 October 2024

DOI: https://doi.org/10.47836/pjtas.47.4.12

*E-mail addresses*:
zaenabnuruljannah@mail.ugm.ac.id (Zaenab Nurul Jannah)
panjono@ugm.ac.id (Panjono)
sigitbintara@ugm.ac.id (Sigit Bintara)
widi.tsm@ugm.ac.id (Tri Satya Mastuti Widi)
adi.tiya.warman@mail.ugm.ac.id (Adi Tiya Warman)
alek002@brin.go.id (Alek Ibrahim)
bayu026@brin.go.id (Bayu Andri Atmoko)
linggalana86@gmail.com (Dayu Lingga Lana)
budisantosa225@gmail.com (Budi Santosa)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

at post-puberty. The body weight of female Sakub sheep has a positive and significant correlation to all linear body sizes, but in different age categories, the correlation weakens with age.

*Keywords*: Body weight, body size, growth pattern, morphometrics characteristics, Sakub sheep

## INTRODUCTION

Sakub sheep is a unique and original local breed raised in Brebes Regency, Indonesia. According to Decree No. 882/KPTS/PK.010/M/12/2022 issued by the Minister of Agriculture of Indonesia (Ministry of Agriculture, 2022), this breed has been officially recognized as a new local sheep in 2022. Despite being an integral part of the local agricultural landscape, Sakub sheep still lacks more specific and scientific exploration. This knowledge gap provides a significant opportunity to enhance the understanding of Sakub sheep characteristics and the potential for local and regional development.

Qualitative characteristics of Sakub sheep based on the Decree of the Minister of Agriculture (Ministry of Agriculture, 2022), include a body shape with high shoulders and a balanced body length (BL) from front to back. The body color comprises white, brown, and black, with wool distributed throughout the body except for the head and feet. A convex shape with long and sideways ears characterizes the facial profile. Moreover, horns may be found in some Sakub sheep, with most females lacking this feature. The shape of the jaw is wide laterally, with the base extending beyond the temples, along with a prominent chest shape and an elongated rounded tail. Based on classification as meat, Sakub sheep have a calm and non-aggressive temperament. Sakub sheep, mainly raised in highland areas, exhibit unique characteristics and become integral to local agriculture (Jannah et al., 2023; Nurasih et al., 2023).

The value of Sakub sheep depends on their ability to adapt to the local environment, characterized by challenging terrain, climatic variations, and limited resources. Although these sheep are suitable for small-scale farming systems, more comprehensive data are required from various aspects, including body size and weight as discernible characteristics, to determine growth patterns (Jannah et al., 2023). The analysis of growth patterns is crucial for assessing sheep's performance and enhancing the quality of meat-producing animals (Afolayan et al., 2006).

The growth pattern in sheep is an essential area of study that includes various aspects such as bone, body frame development, and growth rates. Understanding and implementing strategies to optimize body weight growth in sheep is crucial for maximizing productivity and profitability. In this context, genetic factors play a significant role, as specific genes and genetic variations have been associated with growth traits in sheep (Pasandideh et al., 2020; Wu et al., 2020; Zhang et al., 2013). Marković et al. (2019) focused on the differentiation of sheep breeds based on morphometric characteristics, providing insight into body size and shape variations.

In light of these considerations, this study aimed to investigate the growth patterns and morphometric characteristics of Sakub sheep as a local breed in the Brebes Regency. The results are expected to fill a crucial knowledge gap, contributing to understanding sheep's growth patterns and providing valuable insights into factors influencing body weight, body sizes, and bone development. Additionally, the results are anticipated to provide valuable information on breeding programs, management practices, and selection strategies to improve growth performance and productivity in sheep populations.

## MATERIALS AND METHODS

### Ethical Clearance

This study was approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia (Certification no. 036/EC-FKH/Eks/2022).

### Description of the Study Area

This study was carried out in the highest areas of Sirampog and Paguyangan Districts, Brebes Regency, Central Java, Indonesia, comprising 92.56% of the Sakub sheep populations. The two districts, located in the highlands of mountain slopes, have an altitude of 1342±61.51 above sea level and an excellent horticultural agricultural sector. These districts are characterized by a mean temperature of 23.80±3.00°C with a humidity of 76.40±15.07%, which is low for tropical climates. Meanwhile, light intensity is 2,838.67±554.94 lux, with a relatively low wind speed at 0.03 ± 0.03 m/s, categorized as conducive to various ecosystems. According to the Central Agency on Statistics of Brebes Regency (Badan Pusat Statistik Kabupaten Brebes, 2023), the precipitate in these districts is 3,678 and 4,850 mm/year, respectively. Table 1 shows a description of the agroecological zones of the study areas.

### Animal Management

This study was carried out between May 2022 and June 2023. A total of 195 female Sakub sheep raised by 60 breeders were selected for this study. Farmer data was obtained from the Brebes District Department of Animal Husbandry and Animal Health. Most Sakub sheep were reared intensively in colony pens made of wood by smallholder farmers as a side job with the traditional system. Feeding was carried out two times a day, consisting of forage grass and agricultural

Table 1
*Measurement of environmental conditions in the Sakub sheep development area*

| Variable | Mean±Standard deviation | Minimum | Maximum |
|---|---|---|---|
| Altitude (masl) | 1,342.00±61.51 | 1,271.00 | 1,379.00 |
| Light intensities (lux) | 2,838.67±554.94 | 2,428.00 | 3,470.00 |
| Temperature (°C) | 23.80±3.00 | 20.80 | 26.80 |
| Wind velocity (m/s) | 0.03±0.03 | 0.00 | 0.50 |
| Humidity (%) | 76.40 ±15.07 | 59.00 | 85.10 |

residues such as carrot and cabbage leaves, without concentration and drinking water. Although farmers rarely maintain colony pens, measurements are made when there is a competition to select a superior breed. Farmers also ignore signs of estrus, leading to random mating in colony pens.

## Data Collection

Sheep measurement was obtained through a door-to-door survey, focusing on body weight and body size of 195 healthy non-pregnant female Sakub sheep. Based on the classification, sheep were categorized into eight distinct age groups, following the procedure of Hakim et al. (2019). The groups consisted of 11 neonatal sheep (aged 0–14 days), 71 young sheep (aged 1–2 months), 16 juvenile sheep (aged >2–4 months), 33 adolescent sheep (aged >4–7 months), 26 young adult ewes (aged >7–12 months, with one pair of permanent incisors), 26 adult ewes (aged >12–24 months, with two pairs of permanent incisors), 27 mature ewes (aged >24–36 months, with three pairs of permanent incisors), and 29 older ewes (aged >36–48 months, with four pairs of permanent incisors). The body weight (BW) of Sakub sheep was measured using digital scale (WeiHeng™, China) with a capacity of 200 kg and an accuracy of 0.01 kg. Meanwhile, body size was measured using measuring tape (Butterfly™, Indonesia) and ruler (FHK™, Japan) with an accuracy of 0.1 cm. The parameters observed for body size, as presented in Figure 1, included heart girth (HG), chest width (CW), chest depth (CD), body length (BL), wither height (WH), hip height (HH), and hip width (HW). Measurements were made based on the Food and Agriculture Organization of the United Nations (FAO) procedure (FAO, 2012) with the sheep's parallelogram position.

## Statistical Analysis

Body size data were analyzed descriptively, and the results were compared with those of similar breeds based on the literature.

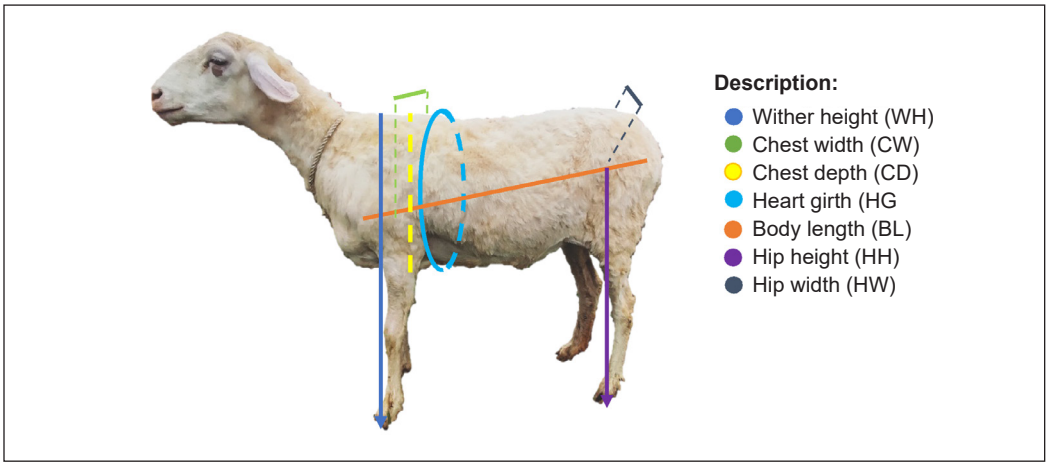



*Figure 1*. The body size observes in female Sakub sheep

Body weight and size data were categorized according to age groups and assessed for normality using the box plot method. Correlation analysis was carried out on body weight and size, which is presented in the heatmap model. Subsequently, all data were analyzed using the SPSS (version 26) and Microsoft Excel (version 16.78).

## RESULTS

### Body Size and Body Weight of Female Sakub Sheep

Table 2 and Figure 2 show the results, statistics, and distribution of body weight and size. The analysis using the box spot method showed that the body weight and size of female Sakub Sheep had a distinctive distribution pattern without outliers in the data. Generally, the standard deviation of female Sakub sheep across different sizes and age groups showed normal outcomes.

Table 2 compares the body weight and size of female Sakub sheep in relation to other local breeds in Central Java. Based on the results, the Sakub sheep's body weight was lower than Batur sheep in the age groups of >12–24, >24–36, and >36–48 months. The body weight of Batur sheep between the ages of 12 to 24 months was 63.12±13.80 kg, while 24 to 48 months had a value of 64.37±12.30 kg. At >12–24 and >24–36 months, the body weight of Sakub sheep was lower compared to Batur sheep. However, at 36–48 months, Sakub sheep had a higher value, while Wosonobo sheep in these age categories had 40.17, 43.84, and 44.59 kg, respectively.

### Body Weight and Body Size Growth Pattern of Female Sakub Sheep

The data were grouped and stratified by age, consisting of 0–14 days, 1–2 months, >2–4 months, >4–7 months, >7–12 months or one pair of permanent incisors, >12–24 months or two pairs of permanent incisors, >24–36 months or three pairs of permanent incisors, and >36–48 months or four pairs of permanent incisors. This stratification was crucial to enable the features of body weight and size, which could vary depending on age. Moreover, age is an essential parameter in the analysis to determine body and size growth patterns.

Based on Table 3 and Figure 2, the body weight of female Sakub sheep showed optimal growth until >36–48 months, reaching an average weight of 47.34 kg. The linear growth pattern of body weight was followed by CW and CD with sizes of 21.76 and 35.35 cm, respectively. Meanwhile, HG, BL, and HH experienced optimal growth at >7–12 months, with dimensions of 80.40 cm, 64.73, and 65.08, respectively. WH growth was also optimal until the age of >12–24 months with a size of 67.27 cm, followed by a gradual body slope towards maturity. However, the HW growth pattern rapidly developed from >7–12 to >12–24 months, with a body size between 13.04 and 15.77 cm.

### Correlation Between Body Weight and Body Size of Female Sakub Sheep

Correlation analysis of female Sakub sheep's body size was carried out on sheep of all ages and in separate age groups. The

Table 2
*The mean ± standard deviation of body weight and size in female Sakub sheep is based on different age groups*

| Traits | Age groups (months) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 (11)* | 1-2 (27)* | >2-4 (16)* | >4-7 (33)* | >7-12 (26)* | >12-24 (26)* | >24-36 (27)* | >36-48 (29)* |
| BW (kg) | 5.10±5.10 | 11.95±2.70 | 15.99±4.55 | 22.22±5.74 | 30.72±4.84 | 37.48±9.38 | 41.32±6.47 | 47.34±7.44 |
| HG (cm) | 40.45±5.70 | 54.37±5.24 | 61.38±7.26 | 69.09±7.59 | 80.04±6.43 | 84.96±7.17 | 86.30±6.25 | 90.21±7.15 |
| CW (cm) | 8.86±1.67 | 12.48±3.13 | 13.31±2.27 | 14.82±2.79 | 15.58±3.63 | 17.46±4.06 | 20.76±5.19 | 21.76±5.69 |
| CD (cm) | 14.45±1.57 | 20.15±2.18 | 23.94±4.40 | 26.27±3.07 | 27.92±4.40 | 30.04±3.70 | 31.74±2.59 | 35.35±3.54 |
| BL (cm) | 34.64±4.06 | 45.81±3.79 | 50.06±5.77 | 58.70±7.47 | 64.73±4.17 | 67.85±5.63 | 69.98±6.91 | 72.55±4.94 |
| WH (cm) | 37.36±3.07 | 50.11±4.57 | 53.63±5.46 | 59.06±5.52 | 63.12±4.02 | 67.27±4.94 | 69.09±4.33 | 70.36±4.14 |
| HH (cm) | 36.55±4.61 | 47.96±4.66 | 52.13±5.73 | 58.24±5.52 | 65.08±5.52 | 66.62±4.15 | 68.50±3.28 | 68.59±2.13 |
| HW (cm) | 7.09±1.30 | 10.33±2.27 | 10.69±2.15 | 12.61±1.92 | 14.23±1.78 | 15.77±2.72 | 17.33±2.10 | 17.91±2.54 |

*Note.* * = The number of samples; BW = Body weight; HG = Heart girth; CW = Chest width; CD = Chest depth; BL = Body length; WH = Wither height; HH = Hip height; HW = Hip width

Table 3
*Comparison mean ± standard deviation of body weight and body size Sakub sheep with other local breed sheep in Central Java, Indonesia*

| Breed/Age (months) | BW (cm) | BL (cm) | HG (cm) | WH (cm) | References |
|---|---|---|---|---|---|
| **Sakub sheep** | | | | | |
| >12-24 | 37.48±9.38 | 67.85±5.63 | 84.96±7.17 | 67.27±4.94 | This study |
| >24-36 | 41.32±6.47 | 69.98±6.91 | 86.30±6.25 | 69.09±4.33 | |
| >36-48 | 47.34±7.44 | 72.55±4.94 | 90.21±7.15 | 70.36±4.14 | |
| **Batur sheep** | | | | | |
| >12-24 | 63.12±13.80 | 68.43±7.45 | 98.35±19.71 | 59.48±6.57 | Ibrahim et al. (2020) |
| >24-48 | 64.37±12.30 | 70.96±5.82 | 102.96±18.75 | 62.08±6.37 | |
| **Wonosobo sheep** | | | | | |
| >12-24 | 40.17 | 67.83 | 79.61 | 60.04 | Hakim et al. (2019) |
| >24-36 | 43.84 | 68.84 | 81.32 | 61.52 | |
| >36-48 | 44.59 | 69.64 | 80.89 | 62.19 | |

*Note.* BW = Body weight; BL = Body length; HG = Heart girth; WH = Wither height

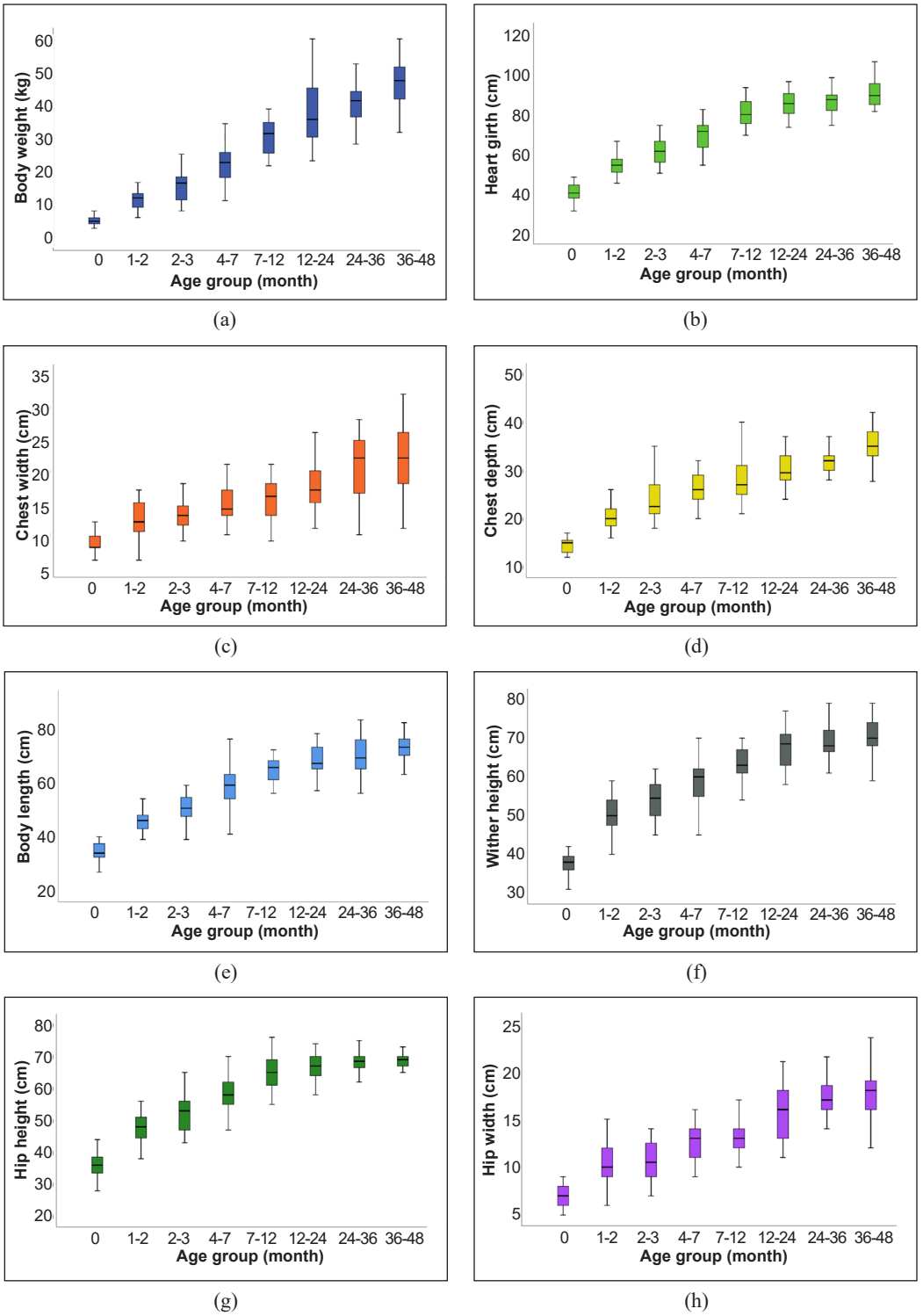



*Figure 2*. Boxplot of body weight and body size of female Sakub Sheep

phenotypic correlation between body weight and body size of female Sakub sheep is presented in Figure 3. The analysis was carried out on 195 sample sizes in various age categories. Besides that, the phenotypic correlation between body size and body weight at different age groups of female Sakub sheep is presented in Figure 4.

Figure 3 shows the results of Pearson correlation between BW to have a highly positive statistical link with all linear body size ($p$<0.01). All measured characteristics were highly and positively statistically correlated.

The layout in Figure 4 establishes a link between body size and body weight in different age groups. Overall, the correlation between body weight and body size weakens with age. It can be seen from the color gradation on the heat map, which fades

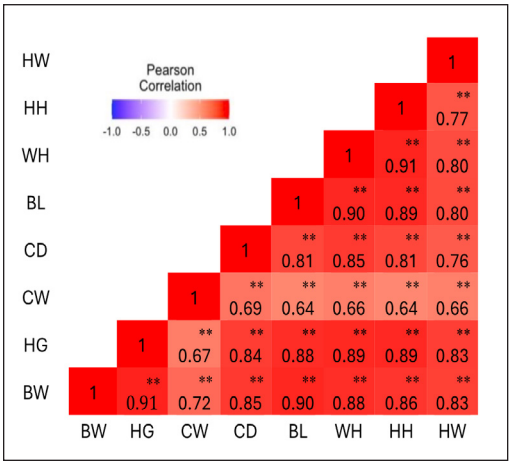



*Figure 3*. Heat map of body weight correlation and body size in female Sakub. The correlation color demonstration is as follows: high correlation is red, mid correlation is white and low correlation is blue
*Note*. BW = Body weight; HG = Heart girth; CW = Chest width; CD = Chest depth; BL = Body length; WH = Wither height; HH = Hip height; HW = Hip width; $^{ns}$ = Not significant; ** = Highly correlation/ Correlation is significant at the 0.01 level (2-tailed); * = Correlated/Correlation is significant at the 0.05 level (2-tailed)

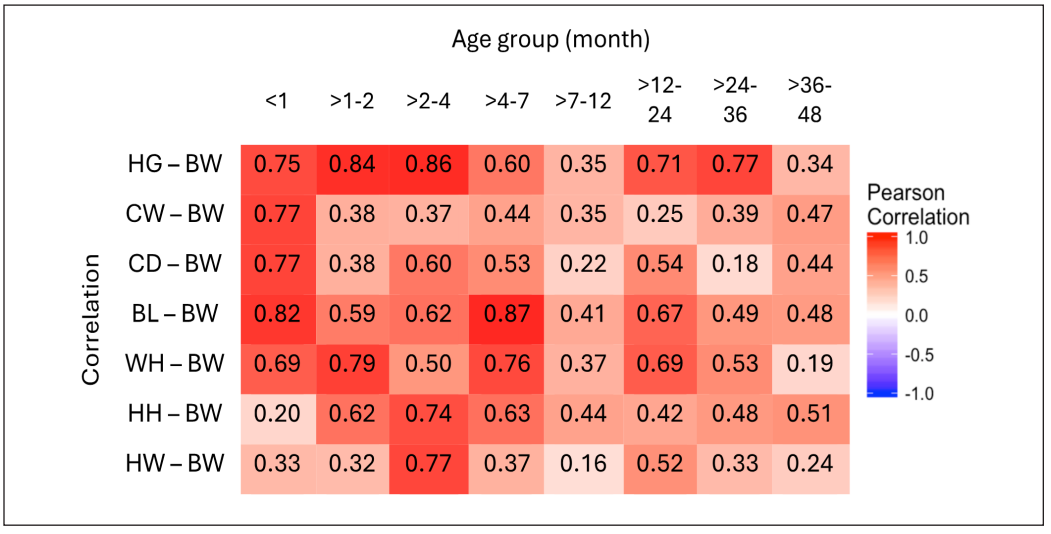



*Figure 4*. Heat map of body weight correlation of body size in female Sakub in different age groups. The correlation color demonstration is as follows: high correlation is red, mid correlation is white and low correlation is blue
*Note*. BW = Body weight; HG = Heart girth; CW = Chest width; CD = Chest depth; BL = Body length; WH = Wither height; HH = Hip height; HW = Hip width; $^{ns}$ = Not significant; ** = Highly correlation/Correlation is significant at the 0.01 level (2-tailed); * = Correlated/Correlation is significant at the 0.05 level (2-tailed)

to the right. Overall, the decrease in the correlation between body size and body weight is significant in the age groups >7–12 and >36–48 months. However, HG, BL, WH, and HH significantly correlate with body weight in almost all age group categories, while CW, CD, and HW are the opposite.

## DISCUSSION

The Minister of Agriculture of Indonesia has issued Decree No. 882/KPTS/PK.010/M/12/2022 (Ministry of Agriculture, 2022), which officially recognized the Sakub sheep as a crossed breed. These sheep are obtained from crossbreeding between local Indonesian and foreign breeds, including Suffolk, Dorper, Texel, and Merino. Other local crossbreeds around Brebes Regency, including Wonosobo, result from crossing thin-tailed and fat-tailed sheep with Texel. As indicated by the name, Wonosobo sheep originated from Wonosobo District (Ministry of Agriculture, 2011b). Furthermore, the Batur sheep, identified in Banjarnegara Regency, is a distinct breed obtained from a cross between local (thin-tailed sheep) and Merino sheep (Ministry of Agriculture, 2011a).

The body size of Batur sheep between the ages of >12–24 and >24–48 months was higher for HG (98.35±19.71 and 102.96±18.75 cm) and BL (68.43±7.45 and 70.96±5.82 cm, respectively), but lower compared to Sakub sheep at the age of >36–48 months. Meanwhile, Batur's WH was lower than Sakub sheep in both age categories (59.48 ± 6.57 and 62.08 ± 6.37 cm, respectively) (Ibrahim et al., 2020), indicating a short and fat body conformation. Wonosobo sheep showed relatively the same BL size as Sakub sheep at the ages of >12–24, >24–36, and >36–48 months, with values of 67.83, 68.84, and 69.64 cm, respectively. However, lower values were obtained for HG at 79.61, 81.32, and 80.89 cm, as well as WH at 60.04, 61.52, and 62.19 cm (Hakim et al., 2019), indicating an extended body conformation, with a smaller front body size.

The body weight of livestock is crucial for assessing dietary requirements. It is associated with reproductive capacity regarding the number of offspring produced, administering medicine doses, evaluating growth, and selecting replacements (Al-Khamaiseh et al., 2020). In this study, the body weight of female Sakub sheep experienced linear growth until the >36–48 months group, reaching 47.34 kg. This result varied significantly from Hakim et al. (2019) regarding the growth pattern of Wonosobo female sheep, where the development curve began to slope at the age of >12–24 and >36–48 months, with a weight of 38.4 and 44.6 kg, respectively. Various factors, including genetics, influence optimal body weight in sheep (Pasandideh et al., 2020; Wu et al., 2020; Zhang et al., 2013), nutrition (Huma & Iqbal, 2019; Jiang et al., 2020; Malik & Muryanto, 2020), management practices (Bhateshwar et al., 2023; Ptáček et al., 2014), and environmental conditions (Bhateshwar et al., 2023).

The development of the vertebrae, including the lumbar, sacral, and thoracic, is

represented by body length in sheep, showing the growth of the skeleton and the vertebral structure. BL's linear growth is followed by CD development, while CW slopes at >36–48 months. CD and CW measurements offer valuable information about the thoracic and ribcage dimensions, showing chest conformation and body shape based on bones and the width of the chest cavity of animals. Similarly, López-Carlos et al. (2010) stated that Dorper sheep had high growth, and canonical discrimination analysis showed superiority in traits related to girth, including HG and CW. Meanwhile, Katahdin and Pelibuey sheep are closely related in terms of SH and hip height (HH) at age different sizes. This phenomenon is attributed to the close relationship with Dorper sheep, indicating the categorization of Sakub sheep in the thick form category. According to Zulfahmi et al. (2016), the growth of internal organs and muscular tissue attached to the shoulder blades affected changes in CD. Suharyati et al. (2023) found that the CD in single pregnancies was less compared to twin births, indicating a connection between performance differences of genetic and environmental factors and the interaction between livestock and their surroundings (Noor, 2010). It shows that several factors, such as nutrient intake, health status, age, genetics, and type of birth, influenced CD and CW.

In this study, HG experienced rapid growth until >7-12 months, followed by a gradual decline. The measurement of HG indicates thoracic circumference, providing insight into chest development and body mass. This observation significantly differs from the previous statement, where HG is considered body size with the closest relationship with body weight. The growth of the chest ribs and the accumulation of thicker flesh impact the chest circumference, as reported by Lake (2016). Bautista-Díaz et al. (2020) stated that abdominal circumference is used to estimate carcasses, indicating the decline in HG growth at >7–12 months. Generally, bone growth slows and stops in old age, while flesh development can continue. Body weight growth is assumed to correlate more positively with abdominal circumference after >7–12 months of age than HG.

BL is a representation of the vertebrae growth, including the lumbar, thoracic, and sacral, which resembles the entire skeletal development and vertebral anatomy of sheep. Meanwhile, WH represents the growth and conformation of the forelegs and upper legs, serving as a critical indicator of body support and structural development in sheep. HH and hip-width (HW) indicate the pelvic structure and hindquarter conformation, showing the functional and anatomical characteristics of the hind limbs as well as the pelvis in sheep. Similar to the HG size, the growth of BL and HH also begins to slope at >7–12 months, while WH slopes at >24–36 months. Although the growth pattern of HW shows irregularities, rapid development is observed from >7–12 to >12–24 months.

Based on these results, body size related to body frame relatively experiences a plateau in adulthood. Meanwhile, other parameters except HG have linear growth

patterns similar to body weight. Compared to Kurniawati et al. (2019) and Trisnawanto et al. (2012), where BL showed a strong correlation with body weight due to its positive correlation with livestock body weight, the growth of BL and WH declined between one and two years of age (Hakim et al., 2019; Ibrahim et al., 2021). Similarly, Tillman et al. (1998) stated that sheep grew rapidly from birth to puberty, followed by deceleration until maturation.

According to the Pearson correlation module (raw), the live weight of Yakansa sheep in the 1–4 years age range was very high ($p$<0.001), which correlated with HG at 0.9. Meanwhile, bone size (HW) was negatively correlated (-0.40) with muscle size (Afolayan et al., 2006). In another study, WH was not included in any trait model correlations with BW in sheep lamb hair (Bautista-Díaz et al., 2020). Zulkharnaim et al. (2016) stated that the BL of goats is an essential factor in stock selection, as higher BL shows a greater potential of bearing twins. However, genetic and nongenetic factors influence animal growth and development curves (Do & Miar, 2019; Kopuzlu et al., 2014) based on breed (Deribe et al., 2023). Pasandideh et al. (2020) and Zhang et al. (2013) further emphasized that genetic factors played an essential role in determining the growth potential of sheep due to the influence of specific genes and genetic variations.

This study showed that body size in female Sakub sheep in the form of CW and CD can continue to grow until maturity. In contrast, the growth pattern in body size related to body frame experiences a gradual slope when entering adulthood. It is attributed to the limitation of bone growth at a certain age, while muscle and fat growth can continue, influencing body size related to muscle. The optimal age for bone growth in sheep varies based on various factors such as breed, genetics, nutrition, and management practices. However, some general patterns and references can provide an idea of bone growth in sheep. Rentsch et al. (2014) stated that bone growth is a complex process influenced by various factors, including hormonal regulation, genetic factors, and environmental conditions. Therefore, the optimal age of bone growth varies between individual animals and breeds (Pearce et al., 2007), indicating that body conformation differs across various sheep.

The correlation between body weight and body size in female Sakub sheep was analyzed using a heat map; the study by Bila (2023) on body measurements of Sussex cows can provide valuable insight. The correlation analysis in this study provides a visual representation of the relationships between different variables and the predicted body weight of the cattle. Adapting this methodology to the context of Sakub sheep could create a heat map illustrating the correlations between different body sizes and body weights in these sheep. Correlation analysis of female Sakub sheep's body size and body weight was conducted on sheep of all ages (Figure 3) and in separate age group categories (Figure 4). The multicollinearity test results for the BW regression model for all HG, CW, CD, BL, WH, HH, and HW

combinations showed a tolerance value >0.10 and the proportion of variance or variance inflation factor (VIF) value <10.00, meaning no multicollinearity in the model (Ibrahim et al., 2021).

The correlation results in Figure 3 demonstrated that BW had an extremely positive, statistically substantial correlation with CD, BL, WH, HH, and HW; nevertheless, it was correlated with CW. For the phenotypic correlation result of female Sakub sheep of all ages, BW had a positive, substantial, and statistical correlation to all linear body measurements. It implies that an increment in size will cause BW to increase and vice versa. The findings support the study of Okpeku et al. (2011), who found positive and highly significant correlations between body weight and biometric traits in West African Dwarf and Red Sokoto goats.

Similarly, Hassen et al. (2012) identified that an increase in heart girth or body length resulted in a corresponding increase in live weight in Ethiopian indigenous goat populations. Furthermore, studies like that of Abera et al. (2014) emphasized the strong relationship between chest girth and body weight in indigenous sheep. It indicates that specific body measurements, such as chest girth, significantly predict body weight in sheep. Moreover, dos Santos Fonseca et al. (2021) highlighted that thoracic perimeter is one of the most correlated body measurements with body weight in goats, consistent with findings in other species like sheep. Additionally, Tuncer et al. (2022) mentioned that measurements such as chest depth and body length are highly correlated with the conformation and growth of sheep. In conclusion, body measurements such as chest girth, heart girth, and thoracic perimeter have been identified as key factors that are positively correlated with body weight in goats and sheep.

The correlation between body weight and body size in different age categories weakens. The color gradation on the heat map fades to the right (Figure 4). Studies have shown that the relationship between body weight and measurements becomes less strong as animals age. Sowande and Sobola (2008) confirmed that the relationship between live weight and body measurements, such as chest girth, in sheep weakens as they age. Furthermore, Abera et al. (2014) highlighted that chest girth, a key body measurement trait, shows the highest correlation with body weight in sheep. However, this correlation weakens as sheep age.

Additionally, studies by Rather et al. (2022) and Zhao et al. (2017) indicated that while certain body measurements like chest width and chest depth significantly correlate with body weight in sheep, this correlation diminishes as the animals grow older. In summary, the correlation between body weight and body size in livestock, particularly sheep, may weaken with age. While certain body measurements are strongly correlated with body weight, this relationship may become less pronounced as animals mature. Understanding these dynamics is crucial for accurate estimation of live weight and effective management practices in livestock farming.

## CONCLUSION

The study showed that the rapid growth of female Sakub sheep, including CW and CD, continued to grow until maturity. In contrast, growth patterns associated with body frames such as BL, WH, HH, and HW showed a rapid increase from birth until puberty, followed by a decline that ceased at maturation. The correlation between body weight and body size of female Sakub sheep of all ages showed that body weight has a positive and significant correlation to all linear body sizes. However, the correlation between body weight and size in different age categories weakens.

## ACKNOWLEDGMENTS

The authors are grateful to the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia for funding this study through the *Pendidikan Magister Menuju Doktor untuk Sarjana Unggul* (PMDSU) program under Grants No. 018/E5/PG.02.00.PL/2023 and contract No. 2206/UN1/DITLIT/Dit- Lit/PT.01.03/2023. Furthermore, the authors are grateful to the Animal Husbandry and Veterinary Agency of Brebes Regency for the invaluable assistance and support from farmers during this study.

## REFERENCES

Abera, B., Kebede, K., Gizaw, S., & Feyera, T. (2014). On-farm phenotypic characterization of indigenous sheep types in Selale area, central Ethiopia. *Journal of Veterinary Science and Technology*, *5*(3), 1000180. https://doi.org/10.4172/2157-7579.1000180

Afolayan, R. A., Adeyinka, I. A., & Lakpini, C. A. M. (2006). The estimation of live weight from body measurements in Yankasa sheep. *Czech Journal of Animal Science*, *51*(8), 343-348. https://doi.org/10.17221/3948-CJAS

Al-Khamaiseh, S. K., Al-Sawalqa, A. G., & Al-Atiyat, R. M. (2020). The effects of doe weight, litter size and sex on kids' birth weight of three goat breeds under a sedentary production system. *Livestock Research for Rural Development*, *32*, 154.

Badan Pusat Statistik Kabupaten Brebes. (2023). *Brebes dalam data 2022: Dinas komunikasi informatika dan statistik Kabupaten Brebes 2023* [Brebes in 2022 data: Brebes Regency informatics and statistics communication services 2023]. Badan Pusat Statistik Kabupaten Brebes.

Bautista-Díaz, E., Mezo-Solis, J. A., Herrera-Camacho, J., Cruz-Hernández, A., Gomez-Vazquez, A., Tedeschi, L. O., Lee-Rangel, H. A., Vargas-Bello-Pérez, E., & Chay-Canul, A. J. (2020). Prediction of carcass traits of hair sheep lambs using body measurements. *Animals*, *10*(8), 1276. https://doi.org/10.3390/ani10081276

Bhateshwar, V., Rai, D. C., & Datt, M. (2023). Heat stress responses in small ruminants under arid and semi-arid regions of western India: A review. *Agricultural Reviews*, *44*(2), 164-172. https://doi.org/10.18805/ag.r-2393

Bila, L., Malatji, D. P., & Tyasi, T. L. (2023). Predicting body weight of South African Sussex cattle at weaning using multivariate adaptive regression splines and classification and regression tree data mining algorithms. *Journal of Applied Animal Research*, *51*(1), 608-615. https://doi.org/10.1080/09712119.2023.2258976

Deribe, B., Tesema, Z., Lakew, M., Zegeye, A., Kefale, A., Shibesh, M., Yizengaw, L., & Belayneh, N. (2023). Growth and growth curve

analysis in Dorper × Tumele crossbred sheep under a smallholder management system. *Translational Animal Science*, *7*(1), txad034. https://doi.org/10.1093/tas/txad034

Do, D. N., & Miar, Y. (2019). Evaluation of growth curve models for body weight in American mink. *Animals*, *10*(1), 22. https://doi.org/10.3390/ani10010022

dos Santos Fonseca, J., de Araujo Pimenta, J. L. L., de Moura, L. S., de Souza, L. C., da Silva, T. L., da Fonseca, C. E. M., & de Oliveira, R. V. (2021). Correlations between body measures with live weight in young male goats. *Acta Scientiarum: Animal Sciences*, *43*(1), e52881. https://doi.org/10.4025/actascianimsci.v43i1.52881

Food and Agriculture Organization of the United Nations. (2012). *Phenotypic characterization of animal genetic resources*. FAO. https://www.fao.org/4/i2686e/i2686e00.pdf

Hakim, F. R., Arifin, M., & Rianto, E. (2019). Growth pattern and productivity of female Wonosobo sheep in Wonosobo District, Central Java Province, Indonesia. In *IOP Conference Series: Earth and Environmental Science* (Vol. 247, No. 1, p. 012044). IOP Publishing. https://doi.org/10.1088/1755-1315/247/1/012044

Hassen, H., Baum, M., Rischkowsky, B., & Tibbo, M. (2012). Phenotypic characterization of Ethiopian indigenous goat populations. *African Journal of Biotechnology*, *11*(73), 13838-13846. https://doi.org/10.5897/AJB12.2092

Huma, Z. E., & Iqbal, F. (2019). Predicting the body weight of Balochi sheep using a machine learning approach. *Turkish Journal of Veterinary and Animal Sciences*, *43*(4), 500-506. https://doi.org/10.3906/vet-1812-23

Ibrahim, A., Artama, W. T., Budisatria, I. G. S., Yuniawan, R., Atmoko, B. A., & Widayanti, R. (2021). Regression model analysis for prediction of body weight from body measurements in female Batur sheep of Banjarnegara District, Indonesia. *Biodiversitas Journal of Biological Diversity*, *22*(7), 2723-2730. https://doi.org/10.13057/biodiv/d220721

Ibrahim, A., Budisatria, I. G. S., Widayanti, R., Atmoko, B. A., Yuniawan, R., & Artama, W. T. (2020). On-farm body measurements and evaluation of Batur sheep on different age and sex in Banjarnegara Regency, Indonesia. *Advances in Animal and Veterinary Sciences*, *8*(10), 1028-1033. https://doi.org/10.17582/journal.aavs/2020/8.10.1028.1033

Jannah, Z. N., Atmoko, B. A., Ibrahim, A., Harahap, M. A., & Panjono. (2023). Body weight prediction model analysis based on the body size of female Sakub sheep in Brebes District, Indonesia. *Biodiversitas Journal of Biological Diversity*, *24*(7), 3657-3664. https://doi.org/10.13057/biodiv/d240702

Jiang, B., Wang, T., Zhou, Y., & Li, F. (2020). Effects of enzyme + bacteria treatment on growth performance, rumen bacterial diversity, KEGG pathways, and the CAZy spectrum of tan sheep. *Bioengineered*, *11*(1), 1221-1232. https://doi.org/10.1080/21655979.2020.1837459

Kopuzlu, S., Sezgin, E., Esenbuga, N., & Bilgin, O. C. (2014). Estimation of growth curve characteristics of Hemsin male and female sheep. *Journal of Applied Animal Research*, *42*(2), 228-232. https://doi.org/10.1080/09712119.2013.842479

Kurniawati, N., Latifah., Maharani, D., Kustantinah., & Hartatik, T. (2019). The effect of birth type on quantitative characteristics in pre-weaned Bligon goats. In *IOP Conference Series: Earth and Environmental Science* (Vol. 387, No. 1, p. 012054). IOP Publishing. https://doi.org/10.1088/1755-1315/387/1/012054

Lake, A. F. (2016). Korelasi PBBH dengan perubahan ukuran linear tubuh pada ternak kambing kacang betina lokal yang diberikan kombinasi hijauan [Correlation of PBBH with changes in linear

body size in local female peanut goats given a combination of forage]. *Journal of Animal Science*, *1*(2), 24-25. https://doi.org/10.32938/ja.v1i02.37

López-Carlos, M. A., Ramírez, R. G., Aguilera-Soto, J. I., Aréchiga, C. F., & Rodríguez, H. (2010). Size and shape analyses in hair sheep ram lambs and its relationships with growth performance. *Livestock Science*, *131*(2-3), 203-211. https://doi.org/10.1016/j.livsci.2010.04.001

Malik, A., & Muryanto. (2020). Kelayakan teknologi pakan fermentasi pada penggemukkan domba batur [Feasibility of fermented feed technology in fattening Batur sheep]. *Jurnal Pengkajian dan Pengembangan Teknologi Pertanian*, *22*(2), 143-150.

Marković, B., Dovč, P., Markovic, M., Radonjić, D., Adakalić, M., & Simčič, M. (2019). Differentiation of some Pramenka sheep breeds based on morphometric characteristics. *Archives Animal Breeding*, *62*(2), 393-402. https://doi.org/10.5194/aab-62-393-2019

Ministry of Agriculture. (2011a). *Keputusan Menteri Pertanian nombor 2915/Kpts/OT. 140/6/2011: Penetapan rumpun domba Wonosobo* [Decree of the Minister of Agriculture number 2915/Kpts/OT. 140/6/2011: Determination of Wonosobo sheep cluster]. Ministry of Agriculture.

Ministry of Agriculture. (2011b). *Keputusan Menteri Pertanian nombor 2916/Kpts/OT. 140/6/2011: Penetapan rumpun domba Batur* [Decree of the Minister of Agriculture number 2916/Kpts/OT. 140/6/2011: Determination of the Batur sheep cluster]. Ministry of Agriculture.

Ministry of Agriculture. (2022). *Keputusan Menteri Pertanian Republik Indonesia nombor 882/KPTS/PK. 010/M/12/2022: Pentapan rumpun domba Sakub* [Decree of the Minister of Agriculture of the Republic of Indonesia number 882/KPTS/PK. 010/M/12/2022: Harvest of Sakub sheep]. Ministry of Agriculture.

Noor, R. R. (2010). *Genetik ternak* [Livestock genetics]. Penebar Swadaya.

Nurasih, A. D., Sumaryadi, M. Y., Hidayah, C. N., Nugroho, A. P., Setyaningrum, A., Haryoko, I., Yuwono, P., & Sodiq, A. (2023). Phenotypic characteristics of Sakub sheep as local livestock genetic resources. *Biodiversitas Journal of Biological Diversity*, *24*(10), 5671-5675. https://doi.org/10.13057/biodiv/d241049

Okpeku, M., Yakubu, A., Peters, S. O., Ozoje, M. O., Ikeobi, C. O. N., Adebambo, O. A., & Imumorin, I. G. (2011). Application of multivariate principal component analysis to morphological characterization of indigenous goats in Southern Nigeria. *Acta Agriculturae Slovenica*, *98*(2), 101-109. https://doi.org/10.2478/v10014-011-0026-4

Pasandideh, M., Gholizadeh, M., & Rahimi-Mianji, G. (2020). A genome-wide association study revealed five SNPs affecting 8-month weight in sheep. *Animal Genetics*, *51*(6), 973-976. https://doi.org/10.1111/age.12996

Pearce, A. I., Richards, R. G., Milz, S., Schneider, E., & Pearce, S. G. (2007). Animal models for implant biomaterial research in bone: A review. *European Cells and Materials*, *13*, 1-10. https://doi.org/10.22203/ecm.v013a01

Ptáček, M., Ducháček, J., Stádník, L., & Beran, J. (2014). Mutual relationships among body condition score, live weight, and back tissue development in meat sheep. *Acta Veterinaria Brno*, *83*(4), 341-346. https://doi.org/10.2754/avb201483040341

Rather, M. A., Bashir, I., Shanaz, S., Alam, S., Shah, R., Hamdani, A., Ahanger, S., & Mir, S. A. (2022). Prediction of body weight from linear body measurements in Kashmiri (*Kashir*) goat. *Bhartiya Krishi Anusandhan Patrika*, *37*(3), 281-284. https://doi.org/10.18805/bkap490

Rentsch, C., Schneiders, W., Manthey, S., Rentsch, B., & Rammelt, S. (2014). Comprehensive histological evaluation of bone implants.

*Biomatter*, *4*(1), e27993. https://doi.org/10.4161/biom.27993

Sowande, O. S., & Sobola, O. S. (2008). Body measurements of west African dwarf sheep as parameters for estimation of live weight. *Tropical Animal Health and Production*, *40*, 433-439. https://doi.org/10.1007/s11250-007-9116-z

Suharyati, S., Pani, T. A., Dakhlan, A., Tantalo, S., & Adhianto, K. (2023). Growth traits of Saburai goats concerning locations in Lampung Province, Indonesia. *Advances in Animal and Veterinary Sciences*, *11*(11), 1840-1845. https://doi.org/10.17582/journal.aavs/2023/11.11.1840.1845

Tillman, A. D., Hartadi, H., Reksohadiprodjo, S., Prawirokusumo, S., & Labdosoekojo, S. (1998). *Ilmu makanan ternak dasar* [Basic animal food science]. Gadjah Mada University Press.

Trisnawanto., Adiwinarti, R., & Dilaga, W. S. (2012). Hubungan antara ukuran-ukuran tubuh dengan bobot badan Dombos jantan [Correlation of body measurements and body weight of male Dombos]. *Animal Agriculture Journal*, *1*(1), 653-668.

Tuncer, S. S., Behrem, S., Arzik, Y., & Kizilaslan, M. (2022). Some morphologic characteristics of Central Anatolian Merino sheep. *Turkish Journal of Agriculture - Food Science and Technology*, *10*(12), 2410-2414. https://doi.org/10.24925/turjaf.v10i12.2410-2414.5293

Wu, M., Zhao, H., Tang, X., Li, Q., Yi, X., Liu, S., & Sun, X. (2020). Novel InDels of *GHR*, *GHRH*, *GHRHR* and their association with growth traits in seven Chinese sheep breeds. *Animals*, *10*(10), 1883. https://doi.org/10.3390/ani10101883

Zhang, L., Liu, J., Zhao, F., Ren, H., Xu, L., Lu, J., Zhang, S., Zhang, X., Wei, C., Lu, G., Zheng, Y., & Du, L. (2013). Genome-wide association studies for growth and meat production traits in sheep. *PLOS One*, *8*(6), e66569. https://doi.org/10.1371/journal.pone.0066569

Zhao, H., He, S., Zhu, Y., Cao, X., Luo, R., Cai, Y., Xu, H., & Sun, X. (2017). A novel 29 bp insertion/deletion (indel) variant of the *LHX3* gene and its influence on growth traits in four sheep breeds of various fecundity. *Archives Animal Breeding*, *60*(2), 79-85. https://doi.org/10.5194/aab-60-79-2017

Zulfahmi, A., Ramdani, D., & Nurmeidiansyah, A. A. (2016). Performa induk domba lokal yang dipelihara secara semi intensif di Kecamatan Pamanukan Kabupaten Subang [Performance of local ewes under semi-intensive system in subdistrict Pamanukan, Subang Regency]. *Students e-Journals*, *5*(4), 1-15.

Zulkharnaim., Syamsu, J. A., Dagong, M. I. A., & Sabile, S. (2016). Peningkatan mutu genetic induk dan calon induk kambing PE prolifik melalui pemanfaatan pakan kulit buah kakao [Improving the genetic quality of prolific PE goats and prospective goats through the use of cocoa shell feed]. *AVES: Junral Ilmu Peternakan*, *10*(2), 1–9. https://doi.org/10.35457/aves.v10i2.185

Pertanika J. Trop. Agri. Sci. 47 (4): 1261 - 1289 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Seawater-induced Salinity Enhances Antioxidant Capacity by Modulating Morpho-physiological and Biochemical Responses in *Catharanthus roseus*

**Dipa Chowdhury[1], Shohana Parvin[1]*, Satya Ranjan Saha[1], Md. Moshiul Islam[2], Minhaz Ahmed[1], Satyen Mondal[1] and Tofayel Ahamed[1]**

[1]*Department of Agroforestry and Environment, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh*

[2]*Department of Agronomy, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh*

## ABSTRACT

Salt stress impedes plant growth and development due to several factors, including the generation of cellular oxidative stressors. This study aimed to assess the impacts of seawater-induced salinity on the plant development, physio-biochemical responses, and antioxidant capacity of *Catharanthus roseus* grown in a variety of seawater (4, 8, and 12 dS/m) for varying durations (60, 90, and 120 days). The experiment was laid out in a randomized complete block design with five replications. The results demonstrated that *C. roseus* successfully endured moderate salinity (8 dS/m) by maintaining plant height, number of leaves, branches, relative water content, and chlorophyll content with a minimum drop in dry biomass (25%) in a time- and dose-dependent approach. Furthermore, greater proline and soluble sugar contents suggested that *C. roseus* possessed enhanced osmoprotective capabilities to counteract osmotic stress caused by salinity. Conversely, all growth indicators decreased significantly at high salinity (12 dS/m). Increased levels of antioxidant enzyme activity catalase and ascorbate peroxidase, phenol and flavonoid, 2,2-diphenyl-1-picrylhydrazyl and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid indicate a coordinated function for antioxidant components in regulating reactive oxygen species (ROS) at low (4 dS/m) and moderate (8 dS/m) salinities. In contrast, excessive salinity

ARTICLE INFO

*Article history:*
Received: 20 December 2023
Accepted: 24 April 2024
Published: 30 October 2024

DOI: https://doi.org/10.47836/pjtas.47.4.13

E-mail addresses:
dipabsmrau@gmail.com (Dipa Chowdhury)
shohana@bsmrau.edu.bd (Shohana Parvin)
satya@bsmrau.edu.bd (Satya Ranjan Saha)
moshiul@bsmrau.edu.bd (Md. Moshiul Islam)
minhaz@bsmrau.edu.bd (Minhaz Ahmed)
satyen@bsmrau.edu.bd (Satyen mondal)
tofayela@gmail.com (Tofayel Ahamed)
*Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

(12 dS/m) led to a burst of ROS, as seen by elevated levels of hydrogen peroxide, malondialdehyde, and electrolyte leakage that greatly reduced total dry matter (72%), especially on days 120. The ion studies on plants subjected to salinity revealed that most $Na^+$ remained in the roots. In contrast, most $K^+$, $Ca^{2+}$, and $Mg^{2+}$ are deposited more firmly in the leaves than in the roots. The findings imply that *C. roseus* may tolerate moderate salinity (8 dS/m) owing to its enhanced antioxidant defense system and osmolytes, which trigger antioxidant enzymes and maintain ionic balance.

*Keywords*: Antioxidant, *Catharanthus roseus*, oxidative stress, proline, seawater

## INTRODUCTION

Salinity is a substantial environmental constraint that significantly affects crop productivity and growth. The continued increase in salinity in arable land brought on by inadequate agricultural methods and climate change is predicted to result in the loss of half of the crop area by the middle of the twenty-first century. According to Akter et al. (2023), the amount of land in Bangladesh impacted by soil salinity continuously grows due to global warming, with a 26.73% increase in salt-prone areas between 1973 and 2009. Around 25–30% of Bangladesh's total arable land is in its salty regions.

Stress due to salt in plants has multitudes of adverse effects, including morpho-physiological, biochemical, and genetic alternations. Osmotic damage and toxicity from ions are the two primary ways that salinity impacts the development and growth of plants (Ali et al., 2022). Osmotic stress brought on by an excessive amount of salt in the root region impairs a root's capacity to absorb water because plant cells are limited in their ability to obtain water by a root signal mediated by abscisic acid (ABA). Secondly, a high salt content within the plant generates ionic toxicity (hyperosmotic stress), which results in cell death — additionally, increased $Na^+$ influx and $K^+$ efflux cause an increased $Na^+/K^+$ ratio in cell membranes, which causes plants to experience osmotic stress (Munns & Tester, 2008). Numerous physiological and biochemical processes, including nutrient intake (such as $K^+$, $Ca^{2+}$, and $Mg^{2+}$) and $CO_2$ assimilation, can be impaired by high salt concentrations, notably higher $Na^+$ concentrations in the transpiration stream of plants (Sarker et al., 2018). The formation of harmful ions modifies how plants interact with water and hinders their photosynthetic pigments, which causes a decrease in transpiration rates, growth, photosynthesis, and biomass production (Dawood et al., 2014).

All these physiological modifications caused by salt in plants exacerbate the overabundance of reactive oxygen species (ROS), which interferes with the regular metabolism of cells and causes oxidative damage by oxidizing lipids, proteins, DNA, and other macromolecules within cells (A. K. Das et al., 2022). To counteract salinity's detrimental effects, plants display various morpho-physiological and molecular

adaptations (Rahman et al., 2017). Plants can generate osmolytes such as soluble sugars, amino acids, and proline (Pro) that shield plant cells from the damaging effects of salt exposure (Sarker et al., 2018). These facilitate adjustment to osmotic pressure, and more of them can increase salt tolerance (Rahneshan et al., 2018). The plants employ a powerful antioxidant defense mechanism to stave off oxidative stress brought on by salinity. Antioxidative enzymes like catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and ascorbate peroxidase (APX); organic compounds (including flavonoids, polyphenols, carotenoids, and ascorbic acid); and antioxidant capacity (2,2-diphenyl-1-picrylhydrazyl [DPPH] and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid [$ABTS^+$]) are crucial for the detoxification of ROS (Wang et al., 2022). The SOD, which changes superoxide into hydrogen peroxide ($H_2O_2$), is regarded as a first line of defense. The $H_2O_2$ is further converted into $H_2O$ by CAT and APX (Islam et al., 2023). Moreover, secondary phytochemicals such as flavonoids, phenols, cyan pigments, tannins, and enzymatic antioxidants boost the total antioxidant capacity of plants by directly neutralizing harmful radicals that are unstable (Azeem et al., 2023; Gengmao et al., 2014). These compounds are also valuable as medicines locally and industrially since they possess a variety of health-improving and disease-prevention qualities.

Nowadays, medicinal plants are significant in sustainable agriculture. Bangladesh is home to medicinal plants that are produced year-round, have lower cultivation expenses, and have less environmental impact. Nevertheless, the shortage of arable land and the fact that most of it is being used to grow food crops to feed her rising population are the results of farmers' unwillingness to cultivate medical plants. Additionally, about 50% of coastal soils eventually become unfit for farming due to their high salt concentration within a year (Dasgupta et al., 2015). The commercial cultivation of several valuable medicinal plants is required to address the growing needs of various ayurveda and pharmaceutical sectors. In this situation, assessing the salt tolerance of potential medicinal plants would be beneficial.

*Catharanthus roseus, a* member of the Apocynaceae family, represents one of the most popular and commercially important medicinal plants globally, including Bangladesh. All the parts of *C. roseus* can be used for medicinal purposes. It has anti-cancer, anti-diabetic, anti-microbial, anti-fungal, antioxidant, anti-ulcer, anti-helminthic, anti-diabetic, hypotensive, hypolipidemic, and is a great therapy for leukemia and chemotherapy of Hodgkin's disease (S. Das & Sharangi, 2017).

Since this plant has potential uses in pharmaceutical industries in addition to local usage, local farmers are interested in growing and gathering it as a commercial crop. However, how applied stress affects this plant's tolerance systems is unknown. Conversely, it would be challenging for new crops to find room due to the declining quantity of arable land. The present study

assesses to get further insight into how seawater influences biomass production, leaf pigmentation, ion buildup, osmotic adjustment, and antioxidant activity of the *C. roseus* plant. Once this is known, it will be feasible to determine whether *C. roseus* farming may be introduced commercially in the saline-affected areas.

## MATERIALS AND METHODS

### Plant Growth and the Design of Experiments

A test crop of the important medicinal plant *C. roseus* was used for this research. The experiment was conducted at the Department of Agroforestry and Environment Research Farm, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur, Bangladesh, from December 2022 to August 2023. Seeds of *C. roseus* collected from the Bangladesh Rural Advancement Committee (BRAC) nursery were sown first in a seedbed for germination. Afterward, uniformly grown one-month-old seedlings with 4–6 leaves were placed into their corresponding pots on December 10, 2022, which had dimensions of 26.50 cm in height by 27.50 cm in a circle. Previously, the pots were filled with 12.86 kg of a soil mixture with a 4:1 ratio of soil to cow dung manure. A randomized complete block design (RCBD) with five replications was employed in this pot investigation. For salinity treatment, seawater with an electrical conductivity (EC) of 49.60 dS/m was used from Cox's Bazar, a district in southern Bangladesh. The tap water was combined with saltwater to create three different salinity levels (4, 8, and 12 dS/m) that could be reasonably applied to *C. roseus* plants.

Unlike controls, after a one-month adaptation phase, two-month-old *C. roseus* plants were irrigated with 500 cc of diluted seawater every three days for 120 days, whereas the controls received tap water. Salinity treatment was administered progressively at a rate of 4, 8, and 12 dS/m to maintain the appropriate concentration to prevent osmotic shock. The treatment was imposed on February 10, 2023. Each plant received a 4 dS/m saline treatment during the first week, except for the control. Apart from control and 4 dS/m treating plants, all plants were treated with 8 dS/m saline at the end of the second week. Control, 4, and 8 dS/m treated rows of plants received the recommended dosage, whereas other plants were treated with a saline solution containing 12 dS/m after the third week. During the salt stress experiment, the average daytime temperature was 20–34°C, and the average nighttime temperature was 14–26°C. The average humidity was 43–87%.

### Measuring Growth Parameters

Data were collected on the plant's height, number of leaves, and number of branches at 60 to 120 days. The total weight of the plant's roots, leaves, and stem was weighed after 120 days as growth markers. The dry weight of a plant's stem and leaves was added to calculate above-ground biomass, also known as shoot dry weight. The roots were thoroughly rinsed with tap water

prior to being measured. Five plants were randomly chosen from each replication of each treatment to evaluate several morpho-physiological and biochemical features at 60, 90, and 120 days after treatment imposition. The first and final data sets were collected on April 10 and June 10, 2023, respectively. All measurements and assays were performed three times for each replicate.

## Physiological Parameters

### *Chlorophyll (Chl) Content and Chlorophyll Stability Index (CSI)*

The chlorophylls (Chl *a*, *b*, and total Chls) and the CSI in fresh *C. roseus* leaves were spectrophotometrically determined using the following equation, which was proposed by Sairam et al. (1997) and Witham et al. (1971), respectively:

Chl *a* (mg/g fresh weight)
= [12.70 (D663) - 2.69 (D645)] × [V/1000 × W]

Chl *b* (mg/g fresh weight)
= [22.90 (D645) - 4.68 (D663)] × [V/1000 × W]

Total Chl (mg/g fresh weight)
= [20.20 (D663) - 8.02 (D645)] × [V/100 × W]

CSI = (Total Chl in stressed leaves/Total Chl in control leaves)

where, D (663, 645) = Optical density of the Chl extract at wavelengths of 663 and 645 nm, V = Final volume (ml) of the 80% acetone with Chl extract, and W = Weight of fresh leaf sample in mg.

### *Estimation of Relative Water Content (RWC)*

According to Tamanna et al. (2023), RWC was measured and calculated using the following equation:

Relative water content (RWC) = (FW - DW) / (TW - DW) × 100

where, FW = Fresh weight, DW = Dry weight, and TW = Turgid weight.

Leaf samples were soaked in distilled water for 24 hr to determine the turgid weight (TW), after which they were blotted dry on paper towels and weighed.

## Biochemical Parameters

### *Proline (Pro) and Soluble Sugar Content*

According to Bates et al. (1973), the amount of Pro in leaves was determined spectrophotometrically using the acid-ninhydrin method. The Pro content is expressed in µmol/g of fresh weight and was determined by utilizing a standard curve. The amount of total soluble sugars was calculated using the Anthrone method (Yemm & Willis, 1954).

### *Oxidative Stress Markers*

A fresh leaf sample weighing 0.5 g was homogenized in 5 ml of 3% ice-cold trichloroacetic acid (TCA, Merck, Germany) and then the mixture was centrifuged for 20 min at 12,879 × *g* and 4°C. The supernatant was then collected, and its amounts of malondialdehyde (MDA) and $H_2O_2$ were

estimated. The extract (0.5 ml) was combined with 1 M potassium iodide (1 ml, Merck, Germany) and potassium phosphate buffer (pH 7, 0.5 ml, Merck, Germany) and incubated for 10 min to ascertain the $H_2O_2$ concentration. The absorbance of the final solution was ascertained at 390 nm according to the Urmi et al. (2023)'s method.

In sealed test tubes containing 0.5 ml of a similar extract, 0.5% 2-thiobarbituric acid (TBA, Sigma-Aldrich, USA) and 0.5 ml of 20% TCA were mixed, and the combination was incubated for 30 min at 95°C in a hot water bath to ascertain the MDA concentration. The samples were submerged in an ice bath, and after the reaction was stopped, they were centrifuged for 10 min at 12,879 × *g*. The absorbances of the resultant supernatant were measured accurately at 532 and 600 nm using a spectrophotometer (Urmi et al., 2023).

A fresh leaf sample (0.5 g) was dissolved in 10 ml of distilled water to assess the electrolyte leakage (EL). The initial readings were obtained using an electric conductivity meter. The samples were sealed and incubated for 30 min in a boiling water bath before the final reading was calculated. The EL percentage was calculated using the Lutts et al. (1996) method.

## Extraction and Assessment of Antioxidant Enzymes

A 0.5 g sample of fresh leaf material was broken up in 5 ml (50 mM) of liquid nitrogen and centrifuged at 35,776 × g at 4°C. The retrieved supernatant was used to perform enzyme reactions.

## Catalase Activity (CAT)

The $H_2O_2$ (15 mM, Sigma-Aldrich, USA) and potassium phosphate buffer (50 mM, Merck, Germany) were combined with enzyme extract (50 L). The mixture's initial absorbance was immediately noted, and the subsequent decline in absorbance was noted after 1 min. The CAT activity was computed utilizing the Noctor et al. (2016)'s method.

## Ascorbate Peroxidase Activity (APX)

A reaction mixture comprising 0.1 mM $H_2O_2$, 0.55 mM ascorbic acid (Sigma-Aldrich, USA), and a phosphate buffer solution (50 mM, pH 7, Merck, Germany) was generated in the absence of light (Nakano & Asada, 1981). Results were recorded as soon as the reaction mixture and enzyme extract (50 L) were mixed.

## Total Antioxidant Capacity

### *Radical Scavenging Activity of DPPH*

Assay for evaluating total antioxidant activity using DPPH radical scavenging (Abdul-Hafeez et al., 2014). One gram of plant samples were homogenized with 5–10 ml of HPLC-grade methanol (Merck, Germany) using a mortar and pestle to a uniform consistency. The samples were centrifuged at 805 × g for 20 min at 40°C, with the supernatants being kept at -20°C for further investigation. After that, 1 ml of the methanolic sample extract was obtained, and 1 ml of the DPPH solution (Sigma-Aldrich, USA) with the equivalent solvent devoid of plant material was taken in another test tube to serve as the control. Each test tube received 3 ml of a 0.2 mM DPPH solution,

and the reaction mixture was then incubated at 20°C for 5 min. The mixture's absorbance was then compared to a blank (methanol) at a wavelength of 517 nm. The amount of DPPH radicals each sample could scavenge was determined using the equation below.

$$\text{Inhibition (I) \%} = Abs_{control} - Abs_{sample} / Abs_{control} \times 100\%$$

The antioxidant activity was calculated using a standard curve, and the antioxidant capacity (µg/g FW) was reported as the equivalent amount of ascorbic acid.

**Radical Scavenging Activity of $ABTS^+$**

The $ABTS^+$ radical scavenging analysis was performed using a modified Cai et al. (2004) methodology. In a nutshell, 2.45 mM potassium persulfate solution (Sigma-Aldrich, USA) and 7 mM $ABTS^+$ stock solution (Merck, Germany) were reacted at a ratio of 0.5:1 to produce $ABTS^+$ radical cations. They were then permitted to stand for 12 to 16 hr at room temperature in the dark. The absorbance of the $ABTS^+$ solution was adjusted by adding 80% ethanol (Merck, Germany) until a reading of 0.700 ± 0.05 was obtained at 734 nm. 0.1 ml of diluted leaf extract was used in a reaction that used 3.9 ml of blue green $ABTS^+$ solution. After 6 min, the solution's absorbance at 734 nm was determined. Trolox standard solution (Merck, Germany) was employed to create standard calibration. The test results, which were run in triplicate, were expressed as Trolox equivalent antioxidant capacity (TEAC, µmol of Trolox equivalent antioxidant capacity based on dry weight, or µmol TEAC/g DW).

$$\text{Inhibition (I) \%} = Abs_{control} - Abs_{sample} / Abs_{control} \times 100\%$$

**Total Phenol and Total Flavonoid Content**

To calculate the total phenolic content (TPC), 0.3 ml of plant extract was combined with 0.15 ml of the 10% Folin-Ciocalteu reagent (Sigma-Aldrich, USA). After 5 min, the reaction mixture was added to a saturated sodium carbonate solution (7.5%) and then incubated for 90 min. After measuring the absorbance at 765 nm, the phenolic content was determined using gallic acid as a reference.

To quantify the total flavonoid concentration (TFC), methanolic extracts (250 µl) were mixed with 10% aluminum chloride (50 µl, Sigma-Aldrich, USA), potassium acetate (50 µl, Sigma-Aldrich, USA), and distilled water (1.4 ml) before being incubated for 40 min. The absorbance was determined at 510 nm using quercetin as a standard.

Abdul-Hafeez et al. (2014) and Zhishen et al. (1999) provided the methodologies used to determine the amounts of total phenol and flavonoid, respectively.

**Ion Content Measurement in Leaves and Roots**

An atomic absorption spectrophotometer (Hitachi, Model: 170-30, Japan) was used to estimate the concentrations of $Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$ ions in the oven-dried samples

of roots and shoots using the techniques described by Mostofa et al. (2015).

### Data Analysis

The data was examined using one-way analysis of variance (ANOVA) using Statistix 10 software. To ascertain whether treatments had statistically significant differences ($p < 0.05$), the least significant difference (LSD) test was performed using Statistix 10 software. Different letters were used to represent these changes. Five biological replications (n = 5) were used to acquire each treatment's values (means ± SEs); the findings are displayed in the tables and figures. Microsoft Excel (version 16.78.3, Vol. License 2019) was used for data calculation, analysis, and subsequent graphical presentation. Minitab was used for the dendrogram, and Origin Pro 9 was used for principal component analysis (PCA).

## RESULTS

### Alternations of the Growth and Biomass of *C. roseus* under Seawater Stress

*Catharanthus roseus* plants exposed to seawater exhibited significant phenotypic interruption, including stunted growth, decolorization of leaves (which turned pale and yellow), and reduction in total fresh weight (TFW) and total dry matter (TDM) upon increasing the degree of seawater for all the sampling dates (Table 1; Figure 1). On day 120, the tallest (108.60 cm) and shortest plants (68.20 cm) were measured in the control and 12 dS/m saline treatments, respectively. Plants exposed to seawater at doses of 4, 8, and 12 dS/m experienced substantial height reductions of 5.5, 9.9, and 19.2% at day 60; 6.8, 14.4, and 34.2% at day 90; and 8.6, 18.0, and 46.4% at day 120, respectively, as compared to the comparable control values (Table 1). Seawater-induced salinity reduced the number of leaves per plant by 5.4, 11.6, and 29.4% at day 90 and 7.4, 16.7, and 36.4% at day 120 when planted under 4, 8, or 12 dS/m salinity levels, respectively. Salt stress brought on by the seawater also significantly negatively affected the number of branches per plant.

The percentage of branch reduction was the lowest (16.3%) at 4 dS/m while the highest (49%) in maximum salinity levels (12 dS/m) at 120 days after treatment application, compared to the unstressed control plants (Table 1). It is apparent from Table 2 that salinity significantly reduced the shoot and root biomass of *C. roseus*. At day 120, seawater with high salinity (12 dS/m) lowered shoot FW and DW by 53.4 and 61%, while root FW and DW decreased by 71.74 and 75.35%, respectively, compared to the corresponding controls (Table 2). Irrigation with seawater having 4 and 12 dS/m salinity reduced TFW by 12.66 and 54.5%, respectively, over their respective controls at day 120 (Table 2). The least and maximum percent reduction of TDM was recorded for the parameter at 4 dS/m (14.65%) and 12 dS/m (72.34%) salinity levels, respectively, as opposed to the control. There is a minimal reduction in TFW and TDM with moderate salinity, 23 and 25%, respectively. Also, with rising seawater treatment, the root/shoot ratio for fresh and dry biomass rose (Table 2).

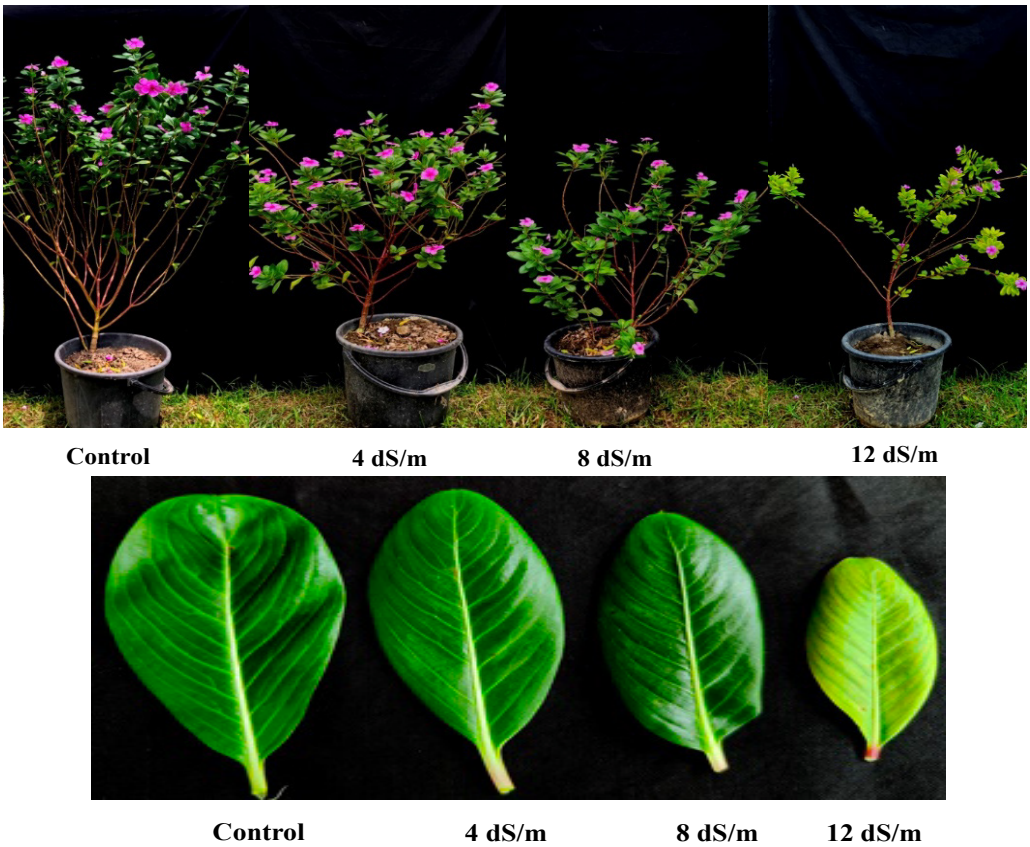



*Figure 1*. Phenotypic appearance of *Catharanthus roseus* plants: control, seawater treatment (4, 8, and 12 dS/m)

Table 1
*Impact of different salt levels generated by seawater on the morphological attributes of* Catharanthus roseus *at different times following treatment imposition*

| Days after treatment initiation | Salinity levels (dS/m) | Plant height (cm) | No. of leaves | No. of branches |
|---|---|---|---|---|
| 60 | Control | 86.20 ± 1.16c | 210.23 ± 2.53a | 37.00 ± 0.37a |
| | 4 | 81.40 ± 0.75c | 201.21 ± 2.24b | 30.00 ± 0.55b |
| | 8 | 77.60 ± 0.93d | 194.83 ± 2.47c | 26.00 ± 0.55c |
| | 12 | 69.20 ± 0.66e | 183.42 ± 2.76d | 23.00 ± 0.60d |
| CV (%) | | 2.03 | 2.70 | 3.24 |
| 90 | Control | 99.60 ± 1.03a | 251.22 ± 1.65a | 44.00 ± 0.37a |
| | 4 | 92.80 ± 0.86b | 237.61 ± 1.72b | 39.00 ± 0.37b |
| | 8 | 85.20 ± 1.32d | 221.84 ± 2.47c | 31.00 ± 0.68c |
| | 12 | 61.60 ± 0.68e | 177.43 ± 2.13d | 25.00 ± 0.37d |
| CV (%) | | 2.83 | 2.05 | 3.18 |

Table 1 *(Continue)*

| Days after treatment initiation | Salinity levels (dS/m) | Plant height (cm) | No. of leaves | No. of branches |
|---|---|---|---|---|
| 120 | Control | 108.60 ± 1.08a | 298.55 ± 1.9a | 55.00 ± 0.40a |
| | 4 | 99.20 ± 0.86b | 276.32 ± 2.4b | 46.00 ± 0.58b |
| | 8 | 89.00 ± 1.58c | 248.62 ± 1.6c | 40.00 ± 0.66c |
| | 12 | 58.20 ± 0.58f | 189.61 ± 2.2d | 28.00 ± 0.45d |
| CV (%) | | 3.99 | 2.32 | 3.20 |

*Note*. Data represents mean ± standard error of 5 independent replicates. The significant differences at $p < 0.05$ are indicated by different letters

Table 2
*Impact of different salt levels generated by seawater on the shoot and root biomass of* Catharanthus roseus *at 120 days following treatment imposition*

| Salinity level (dS/m) | Shoot fresh weight (g) | | Root/ Shoot | Root fresh weight (g) | TFW (g) | Shoot dry weight (g) | | Root dry weight (g) | Root/ Shoot | TDM (g) |
|---|---|---|---|---|---|---|---|---|---|---|
| | Leaf fresh weight (g) | Stem fresh weight (g) | | | | Leaf dry weight (g) | Stem dry weight (g) | | | |
| Control | 90.54 ± 0.46a | 375.40 ± 2.13a | 0.15 ± 0.32c | 70.90 ± 0.84a | 530.84 ± 1.60a | 37.78 ± 0.44a | 130.94 ± 0.86a | 31.00 ± 0.84a | 0.18 ± 0.12c | 204.72 ± 0.50a |
| 4 | 82.24 ± 0.63b | 317.11 ± 0.87b | 0.16 ± 0.24 | 64.26 ± 0.76b | 463.61 ± 1.50b | 33.22 ± 0.46b | 115.70 ± 0.66b | 28.80 ± 0.76b | 0.19 ± 0.22b | 174.72 ± 1.10b |
| 8 | 73.78 ± 0.51c | 285.65 ± 1.48c | 0.17c ± 0.56b | 59.76 ± 0.61c | 409.19 ± 2.44c | 24.42 ± 0.37c | 103.62 ± 0.44c | 24.58 ± 0.61c | 0.19 ± 0.15b | 152.62 ± 0.16c |
| 12 | 59.62 ± 0.51d | 151.71 ± 0.89d | 0.22 ± 0.21a | 45.97 ± 1.17d | 241.30 ± 1.57d | 10.42 ± 0.57d | 46.81 ± 0.37d | 11.38 ±1.17d | 0.20 ± 0.23a | 56.61 ± 0.84d |
| CV (%) | 3.64 | 4.46 | 3.33 | 3.45 | 1.15 | 3.36 | 1.71 | 2.46 | 2.19 | 4.31 |

*Note*. Data represents mean ± standard error of 5 independent replicates. The significant differences at $p < 0.05$ are indicated by different letters. TFW = Total fresh weight; TDM = Total dry matter

## Alteration in the Physiological Responses of *C. roseus* to Seawater Stress

The amounts of Chl *a*, Chl *b*, and total Chl in the leaves of *C. roseus* plants treated with various salinities induced by saltwater decreased progressively and noticeably (Table 3). The Chl *b* content of leaves rose with the passage of time up to 90 days under salinity treatments of 4 and 8 dS/m and, after that, progressively declined. After applying low (4 dS/m), moderate (8 dS/m), and high salinity (12 dS/m), the Chl *a* content was reduced by 12, 25, and 43%, the Chl *b* content by 6.7, 11.5, and 32%, and the total Chl content by 10.5, 22, and 41% at day 120. Under severe salt stress (12 dS/m) at day 120, the reduction of Chl *a* (by 43%) was larger than the reduction of Chl *b* (by 32%), resulting in a noticeably lower level of overall Chl content (41%).

As evident from Table 3, seawater stress had a negative effect on the Chl stability index (CSI) of the *C. roseus* plant. The CSI dropped 33.66%, whereas the salt level went up from 4 to 12 dS/m at day 120. Under stress from seawater, the RWC of *C. roseus* leaves was comparatively higher early in the growth cycle than at a later stage. RWC was found to be decreased by 5.31, 16.43, and 25.76% at day 90 and 7.7, and 22.90 and 35.24% at day 120 under 4, 8, and 12 dS/m salinity levels, respectively, in contrast to the relative control value.

Table 3
*Impact of different salt levels generated by seawater on chlorophyll* a *(Chl* a*), chlorophyll* b *(Chl* b*), total chlorophyll (Chls), chlorophyll stability index (CSI), and relative water content (RWC) of* Catharanthus roseus *leaves at different times following treatment imposition*

| Days after treatment imposition | Salinity levels (dS/m) | Chl *a* (mg/g FW) | Chl *b* (mg/g FW) | Total Chl (mg/g FW) | CSI (%) | RWC (%) |
|---|---|---|---|---|---|---|
| 60 | Control | 3.22 ± 0.02a | 0.95 ± 0.06a | 4.17 ± 0.14a | - | 90.96 ± 0.34a |
| | 4 | 2.95 ± 0.01b | 0.98 ± 0.04b | 3.93 ± 0.21b | 94.24 ± 0.33b | 87.44 ± 0.39b |
| | 8 | 2.90 ± 0.02c | 1.04 ± 0.02c | 3.94 ± 0.15c | 94.48 ± 0.25c | 79.95 ± 0.42c |
| | 12 | 2.68 ± 0.01d | 0.81 ± 0.01d | 3.49 ± 0.23d | 83.69 ± 0.74d | 72.74 ± 0.23d |
| 90 | Control | 3.38 ± 0.02a | 0.98 ± 0.01a | 4.36 ± 0.12a | - | 91.78 ± 0.23a |
| | 4 | 3.11 ± 0.01b | 1.08 ± 0.03b | 4.19 ± 0.18b | 96.10 ± 0.23b | 86.90 ± 0.33b |
| | 8 | 2.75 ± 0.04c | 1.12 ± 0.01b | 3.87 ± 0.25c | 88.76 ± 1.37c | 76.70 ± 0.19c |
| | 12 | 2.31 ± 0.01d | 0.79 ± 0.03c | 3.10 ± 0.14d | 71.10 ± 0.67d | 68.14 ± 0.27d |
| 120 | Control | 3.54 ± 0.01a | 1.04 ± 0.01a | 4.58 ± 0.11a | - | 92.65 ± 0.22a |
| | 4 | 3.13 ± 0.03b | 0.97 ± 0.03b | 4.10 ± 0.32b | 89.51 ± 0.20 b | 85.51 ± 0.21b |
| | 8 | 2.66 ± 0.01c | 0.92 ± 0.03c | 3.58 ± 0.16c | 78.16 ± 1.45c | 71.46 ± 0.26d |
| | 12 | 2.01 ± 0.01d | 0.71 ± 0.10 d | 2.72 ± 0.12d | 59.38 ± 0.60d | 60.00 ± 0.27e |
| CV (%) | | 5.10 | 3.81 | 2.16 | 3.41 | 3.77 |

*Note*. Data represents mean ± standard error of 5 independent replicates. The significant differences at $p < 0.05$ are indicated by different letters

## Alternations in the Biochemical Responses of *C. roseus* to Seawater Stress Soluble Sugar and Pro Content

To comprehend the mechanisms behind osmotic adjustment, the quantities of Pro and soluble sugars in *C. roseus* plants at various salinity levels were examined. The seawater stress caused a considerable rise in Pro and soluble sugar levels at all sampling dates (Figure 2). *C. roseus* rapidly increased leaf Pro as salinity increased from low to moderate and high salinities (8 and 12 dS/m) (216 and 241%) at day 90 and statistically remained constant (301 and 309%) at day 120, respectively, as compared to control. As opposed to controls, soluble sugars steadily increased at days 60, 90, and 120 under moderate (8 dS/m) (98, 143, 225%) and high (12 dS/m) (133, 224, 306%) salinity conditions, respectively (Figure 2).

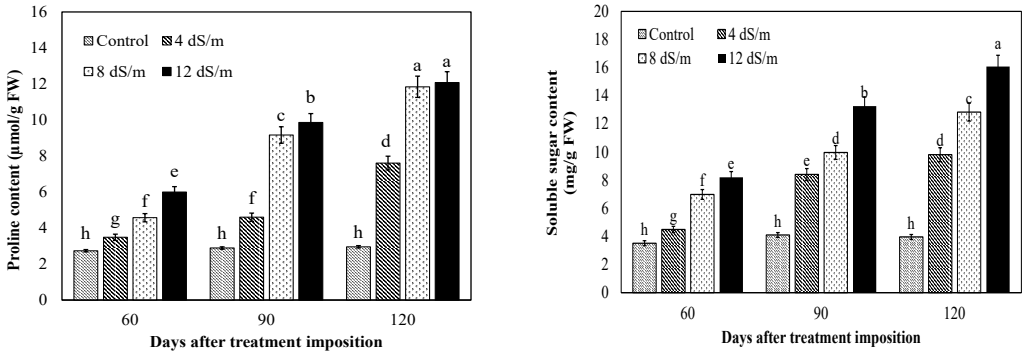



*Figure 2*. Impact of different salt levels generated by seawater on the (a) proline (Pro) and (b) soluble sugar content of *Catharanthus roseus* at different times following treatment imposition

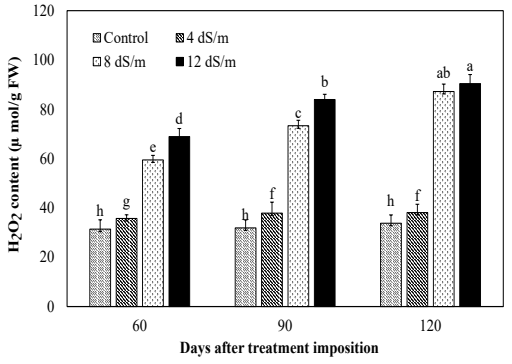
*Note*. Means and standard errors are displayed using bars (n = 5). The significant differences at $p < 0.05$ are indicated by different letters

## Generation of Oxidative Stress Indicators

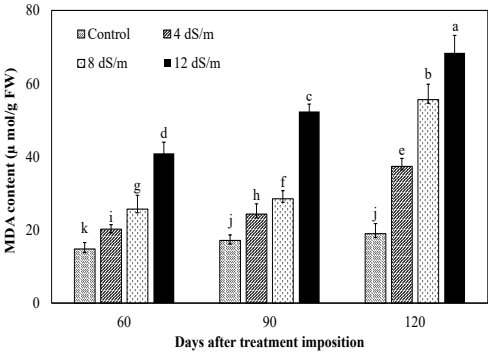
Oxidative stress indicators such as $H_2O_2$, MDA, and EL increased with salinity, reaching the highest levels at 12 dS/m salinity at day 120 (Figure 3). The $H_2O_2$ concentration increased significantly at moderate and severe salinities (8 and 12 dS/m) by 89, 119% at day 60; 129, 163% at day 90; and 158, 167% at day 120, respectively, compared to the relative control value, whereas at low salinities (4 dS/m), there were no discernible changes in $H_2O_2$ contents (Figure 3a). Mild (4 dS/m) and moderate salinity (8 dS/m) had little effect on MDA levels at days 60 and 90. However, after being exposed to saltwater at concentrations of 4, 8, and 12 dS/m for 120 days, there was an apparent rise in MDA content (175, 204, and 258%) compared to the control (Figure 3b). The EL concentration was not pointedly impacted by low or moderate salinity at any of the sampling dates; however, the percentage increase was only found to be highest at high salinity (by 194, 251, and 622% at days 60, 90, and 120, respectively) compared to control (Figure 3c).

## Alternations of Antioxidant Compounds, Capacity, and Enzymes under Seawater Stress

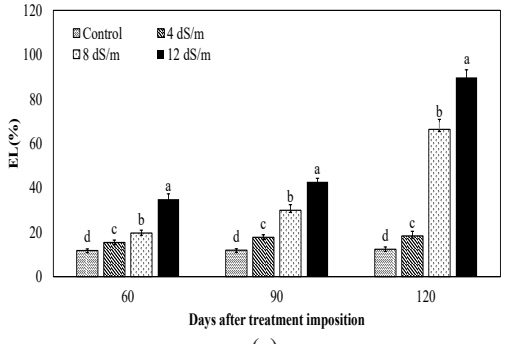
The antioxidant defense mechanism, which is composed of enzymes that are antioxidant (CAT and APX), antioxidant ability (DPPH and $ABTS^+$), and antioxidant substances (total phenols and flavonoids), has significantly increased upon increasing seawater stress (Table 4; Figure 3). The activity of CAT and APX progressively rose as salt concentrations and exposure periods were increased (Figures 3d and 3e). On days 60 and 90, CAT increased at moderate (40 and 70%) and high (69, 139%) salinities (Figure 3d), whereas APX (99 and 138%) significantly increased only at high salinities, respectively, in comparison to the control (Figure 3e). After



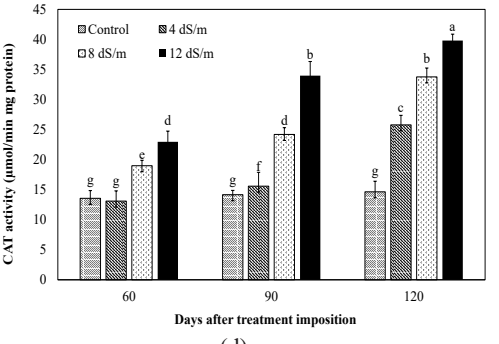

(a)



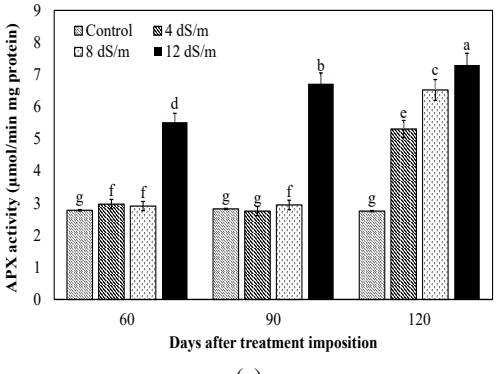

(b)




(c)




(d)




(e)

*Figure 3*. Impact of different salt levels generated by seawater on (a) hydrogen peroxide ($H_2O_2$), (b) malondialdehyde (MDA), (c) electrolyte leakage (EL) content, (d) catalase (CAT), and (e) ascorbate peroxidase (APX) activity of *Catharanthus roseus* at different times following treatment imposition

*Note*. Means and standard errors are displayed using bars (n = 5). The significant differences at $p < 0.05$ are indicated by different letters

the seawater exposure time was prolonged for an additional 30 days, CAT activity was elevated by 76, 130, and 171% and APX activity by 93, 137, and 165% at salinity indices of 4, 8, and 12 dS/m, respectively, as opposed to control. There was no substantial variation in DPPH and $ABTS^+$ activities at low salinity; however, at both moderate and high salinity (8 and 12 dS/m), the activities of DPPH (138 and 201%) and $ABTS^+$ (78 and 123%) considerably increased on day 90 as compared with control. Alternatively, DPPH activities were further increased by 198, 234%, and $ABTS^+$ 100, 192%, at intermediate (8 dS/m) and severe (12 dS/m) salinity levels on day 120, respectively, in contrast to control. The increasing rate of phenol content was found to be 102.28,

280.7, and 275.4% of control under 4, 8, and 12 dS/m salinities, respectively, indicating that the rate of accumulation of TPC was higher at the 8 dS/m treatment level at day 120. The increment of TFC displayed the greatest at the severe salinity level (12 dS/m); the TFC was 100.54 and 105.94% higher at days 90 and 120, respectively, as opposed to salt-free control plants.

Table 4
*Impact of different salt levels generated by seawater on total antioxidant activity, TFC, and TPC of* Catharanthus roseus *leaves at different times following treatment imposition*

| Days after treatment imposition | Salinity levels (dS/m) | Total antioxidant activity (DPPH) (µg/g FW) | Total antioxidant activity ($ABTS^+$) (µg/g FW) | Total flavonoid content (TFC) (mg of quercetin/g FW) | Total phenol content (TPC) (mg/g GAE FW) |
|---|---|---|---|---|---|
| 60 | Control | 22.95 ± 0.40g | 40.22 ± 1.94f | 1,242.57 ± 7.02ef | 228.97 ± 5.45h |
| | 4 | 23.09 ± 0.75g | 44.84 ± 1.66f | 1,362.43 ± 14.57e | 321.43 ± 3.92g |
| | 8 | 38.74 ± 0.62e | 61.67 ± 1.62e | 1,791.83 ± 9.50d | 383.43 ± 8.98f |
| | 12 | 46.76 ± 0.49d | 80.33 ± 2.91c | 2,023.97 ± 13.38c | 552.97 ± 9.85d |
| 90 | Control | 22.36 ± 0.41g | 42.19 ± 1.64f | 1,304.71 ± 6.59e | 210.71 ± 4.40h |
| | 4 | 26.29 ± 0.29f | 45.28 ± 3.74f | 1,558.19 ± 11.45d | 331.19 ± 2.17g |
| | 8 | 53.38 ± 0.48c | 75.32 ± 2.84d | 1,904.76 ± 6.83c | 440.76 ± 8.76e |
| | 12 | 67.36 ± 0.41b | 94.18 ± 2.44b | 2,616.59 ± 11.22b | 689.59 ± 8.20c |
| 120 | Control | 23.20 ± 0.37g | 44.21 ± 2.01f | 1,467.40 ± 7.28e | 207.00 ± 5.09h |
| | 4 | 27.98 ± 0.33f | 68.76 ± 1.15d | 1,602.73 ± 5.45d | 418.73 ± 6.03ef |
| | 8 | 69.36 ± 0.74b | 88.52 ± 1.53c | 2,125.82 ± 15.50c | 788.11 ± 4.73a |
| | 12 | 77.60 ± 0.57a | 129.43 ± 5.81a | 3,022.11 ± 13.69a | 777.22 ± 8.62b |
| CV (%) | | 4.56 | 3.91 | 5.97 | 5.33 |

*Note*. Data represents mean ± standard error of 5 independent replicates. The significant differences at $p < 0.05$ are indicated by different letters

## Mineral Content

The study examined the levels of $Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$ in the roots and leaves to comprehend *C. roseus*'s ion homeostasis strategy on day 120 of the varied seawater treatments. It was clear that saline stress had a remarkable effect on $Na^+$ content in leaves and roots and that this effect grew with increasing salinity. However, the content of $Na^+$ was found to be lower in leaves than in roots. Figure 4a demonstrates that treatment of *C. roseus* plants with various seawater levels (4, 8, and 12 dS/m) significantly increased the content of $Na^+$ (68, 74, and 78% in roots and 54, 61, and 74% in leaves, respectively). Leaf $K^+$ concentration increased (7.5%) in 4 dS/m salinity treatments, despite being statistically similar to the control, before starting to drop (Figure 4b). It is pertinent to note that leaves have been shown to have lower $Na^+$ concentrations and higher $K^+$ concentrations than roots, indicating that *C. roseus* has a greater ability to withstand salt. It also noticed a considerable drop in the amounts of $Ca^{2+}$ and $Mg^{2+}$ in the

leaves and roots of seawater-stressed plants compared to control plants grown without access to seawater (Figures 4c and 4d). In addition, plants stressed by seawater had substantially larger amounts of $Ca^{2+}$ and $Mg^{2+}$ in their leaves than in their roots. The $K^+/Na^+$ ratios were assessed and found that when salt levels steadily increased, the ratios in both leaves and roots tended to trend lower. Notably, leaves, as opposed to roots, showed greater $K^+/Na^+$ ratios (Figure 4e). *Catharanthus roseus* maintained larger amounts of $K^+$, $Ca^{2+}$, and $Mg^{2+}$ in leaves and modified its ion homeostasis during salt stress because of the retention of $Na^+$ in roots.

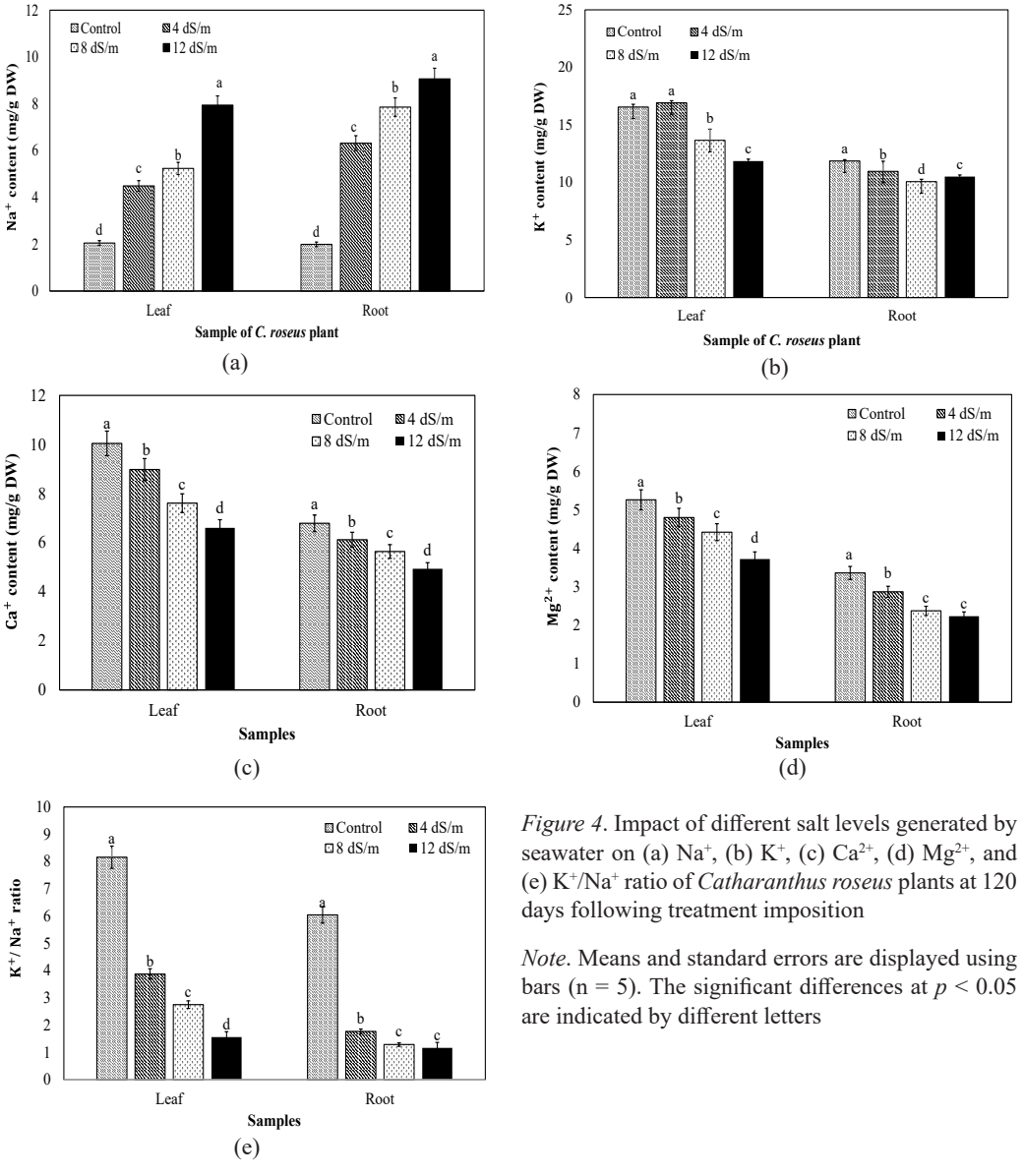


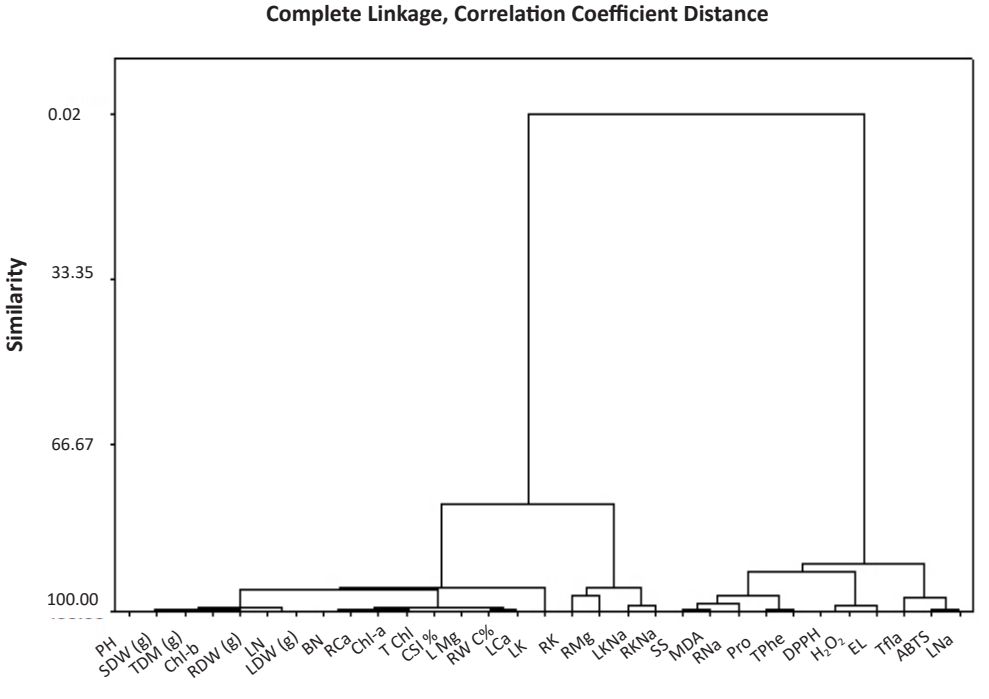

*Figure 4*. Impact of different salt levels generated by seawater on (a) $Na^+$, (b) $K^+$, (c) $Ca^{2+}$, (d) $Mg^{2+}$, and (e) $K^+/Na^+$ ratio of *Catharanthus roseus* plants at 120 days following treatment imposition

*Note*. Means and standard errors are displayed using bars (n = 5). The significant differences at $p < 0.05$ are indicated by different letters

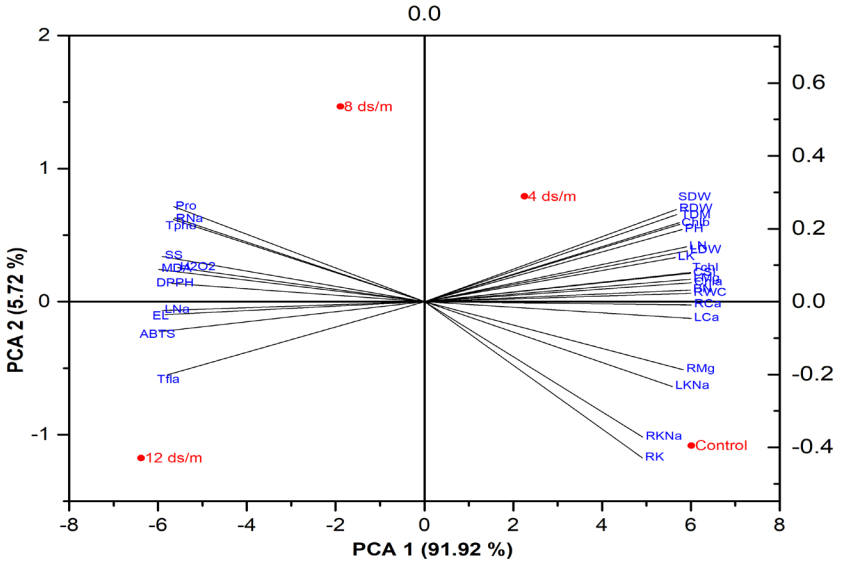
**Data from Morpho-physiological and Biochemical Processes are Displayed Using a Dendrogram and PCA**

To evaluate the gathered data rapidly, a clustered dendrogram was generated. Four clusters are generated in the dendrograms A (total flavonoid [Tfla], $ABTS^{+}$, leaf $Na^{+}$ [$LNa^{+}$]), B (soluble sugar [SS], MDA, root $Na^{+}$ [$RNa^{+}$], Pro, total phenol [TPhe], DPPH, $H_2O_2$), C (root $K^{+}$ [$RK^{+}$], root $Mg^{2+}$ [$RMg^{2+}$], leaf $K^{+}/Na^{+}$ [$LK^{+}/Na^{+}$], root $K^{+}/Na^{+}$ [$RK^{+}/Na^{+}$] ratio), and D (plant height [PH], shoot dry weight [SDW], total dry matter [TDM], Chl *b*, root dry weight [RDW], leaf number [LN], leaf dry weight [LDW], branch number [BN], root $Ca^{2+}$ [$RCa^{2+}$], Chl *a*, total Chl [TChl], CSI, leaf $Mg^{2+}$ [$LMg^{2+}$], leaf $Ca^{2+}$ [$LCa^{2+}$], leaf $K^{+}$ [$LK^{+}$]). Cluster A was strongly linked with cluster B, showing a similarity of about 90. On the other hand, the C and D clusters are linked, showing a similarity of about 80. Among the four clusters, variables are very strong in the D cluster, followed by B, A, and C. Variables from clusters A and B revealed an increased trend when plants were subjected to 8 and 12 dS/m of salt stress compared to the control treatment employing seawater-free circumstances. As opposed to untreated control plants, cluster C and D variables obtained at 4, 8, and 12 dS/m of salt stress showed declining trends (Figure 5a).

PCA was used to analyze the correlations between the treatments and the different variables (Figure 5b). When combined, the PCA1 and PCA2 scores explained 97.64%




(a)




(b)

*Figure 5*. (a) Dendrogram and cluster analysis of growth, physiological, and biochemical traits of *Catharanthus roseus* plant; (b) principal component analysis (PCA) was employed to make links between treatments and variable levels clearer

*Note*. The variables included PH (plant height), LN (leaf number), BN (branch number), TFW (total fresh weight), TDM (total dry matter), RWC (relative water content), $LCa^{2+}$ (leaf calcium content), $RCa^{2+}$ (root calcium content), Chl *a* (chlorophyll *a*), Chl *b* (chlorophyll *b*), TChl (total chlorophyll), $LMg^{2+}$ (leaf magnesium content), $RMg^{2+}$ (root magnesium content), $LK^{+}$ (leaf potassium content), $RK^{+}$ (root potassium content), SS (soluble sugar), LK/Na (leaf K/Na ratio), RK/Na (root $K^{+}/Na^{+}$ ratio), CSI (chlorophyll stability index), MDA (malondialdehyde), $LNa^{+}$ (leaf sodium content), $RNa^{+}$ (root sodium content), Pro (proline), TPhe (total phenol), Tfla (total flavonoid), and $H_2O_2$ (hydrogen peroxide), respectively

of the variability in the data. A PCA biplot showed that PCA1 contributed positively to physiological traits like $H_2O_2$, Pro, TPhe, DPPH, SS, and $RNa^{+}$, accounting for around 91.92% of the total variability, and negatively to factors like $LNa^{+}$, EL, $ABTS^{+}$, and Tfla, accounting for 5.72% of the total variation.

## DISCUSSION

### Effect of Seawater-induced Salinity on the Growth Parameters

Plants expend enough energy on various metabolic functions to adapt to environmental challenges. Salt stress has a major impact on plant metabolism and development. According to the present investigation, seawater reduced the growth performance of *C. roseus* by interfering with various traits essential to its growth. These metrics were plant height, the quantity of leaves, branches and shoots, and root FW and DW, which were strongly affected by increasing the levels of salinity (Tables 1 and 2; Figure 1). A higher accumulation of sodium chloride in the cytosol and cell membrane of *C. roseus* leaves may result in fewer leaves. Concurrently, the cell sap

on the leaves might not have been able to retain more salt at the same time, which led to a decrease in the salt content of the cells and a rapid withering of the leaves (Haddadi et al., 2016).

In agreement with our findings, Obaidullah et al. (2022) and S. Das et al. (2024) reported that elevated salinity levels reduced the number of leaves in *Vitex negundo* and *Justicia adhatoda*. Likewise, it was also evident that rising salinity had a detrimental effect on the emergence of new buds. The decline in branches was brought on by steadily increasing salinity, which is corroborated by studies on *J. adhatoda* by Obaidulahah et al. (2022), *Zea mays* by Hassanein et al. (2009), and *Withania somnifera* by Jaleel et al. (2008). According to Table 2, the present investigation illustrates a negative correlation between seawater stress and TFW and TDM, which is in line with the findings of Azeem et al. (2023) and Li et al. (2022). A general drop in DW has been shown in all *C. roseus* plant tissues under salt stress; however, this decrease is particularly noticeable in the aerial section of the plant. This drop in shoot biomass of seawater-induced *C. roseus* plants could be attributed to insufficient nutrient availability in the growth medium, a slower rate of water entry into the plants, a decline in photosynthetic output, and a suppression of $CO_2$ supply.

It can also be explained that the considerable drop in TFW and TDM of *C. roseus* can be attributed to reduced photosynthesis, a stagnant or reduced mobilization of reserve foods, and an interruption of plant cell division (Alam et al., 2015). Extreme salinity significantly impairs cell proliferation and growth, meristematic function, carbon sequestration, the distribution of photoassimilation among roots, stems, and leaves, as well as both nutrients and water absorption (Pan et al., 2016; Rahman et al., 2017), all of which lead to decreased growth performance, as was observed in this experiment. In accordance with previous reports, certain species can withstand moderate salinity, exhibiting growth reductions ranging from 25 to 50% (Cassaniti et al., 2012). *C. roseus* can withstand 8 dS/m salt successfully by retaining its biomass accumulation in both the root and the shoot, only experiencing a small decrease in the overall fresh (23%) and dry (25%) biomass (Table 2) compared to the respective control. The finding is also supported by similar research on *V. negundo* and moringa (Bekka et al., 2022; S. Das et al., 2024).

However, TFW (54%) and TDM (72%) of *C. roseus* dramatically decreased at severe salinity (12 dS/m). Excessive salt content in the rooting media causes a physiological drought by lowering the water content of the tissue and limiting the amount of water available before it becomes ion-toxic. A plant may utilize half of its energy to regulate metabolic processes and the remaining to grow and produce biomass (Uematsu et al., 2012). The study suggests that moderate salinity (8 dS/m) may reduce the overall amount of energy generated by photosynthesis and redirect a significant portion of that energy into stress-regulatory processes rather than development.

**Physiological Traits Caused by Seawater Salinity and Their Impact**

It was noticed that *C. roseus* plants exhibit a decrease in photosynthetic pigments, primarily Chl *a* and Chl *b*, essential to photosynthesis, with an increment in seawater concentration and exposure time (Table 3). Chl degradation was observed more than synthesis under salinity stress. It can be explained by the development of proteolytic enzymes like chlorophyllase, which degrade Chl while also harming the photosynthetic apparatus (Isayenkov & Maathuis, 2019), reduce the plant photosynthesis process (Mafakheri et al., 2010), and prevent the buildup of accumulated ions (Jaleel et al., 2008). The study results are in accordance with findings from research on pepper and common beans by Abdelhamid et al. (2013) and Hand et al. (2017), respectively.

The decrease in total Chl concentration, especially at moderate salinity (8 dS/m) levels, suggests a way to prevent excessive ROS generation and shield the photosynthetic machinery from salt-induced photodamage (Farooq et al., 2022). *C. roseus* also had a similar pattern of pigment loss when exposed to salt (Table 3). According to Acosta-Motos et al. (2017), salt tolerance of the plant affects the Chl content, even though it decreases during salt stress. The disruption of the photosynthetic mechanism, the instability of pigment-protein complexes, the dysfunction of pigments, and structural damage to the light-harvesting complex may all be contributing factors to the decline in total Chls (Geissler et al., 2009). Additionally, excessive salt concentrations cause chloroplasts to generate ROS that breaks the multiple bonds of fatty acids that are unsaturated, damages the membrane of the chloroplast, and lets Chl spill out of the thylakoids (Sun et al., 2010). Our results also revealed that Chl *a* is more sensitive to salinity than Chl *b*, in accordance with past investigations of Gururani et al. (2015). They reported that the most abundant and necessary part of the light-harvesting complex is Chl *a*, while Chl *b* serves as a supplemental pigment and incidentally aids in photosynthesis by transmitting photons to Chl *a*. The prevailing consensus is that the faster breakdown of their pigment, impeded synthesis, and rapid plastid degradation are the causes of these pigments' decline under salt stress (Chen, 2014). Thus, *C. roseus* may have survived the salt-induced loss of photosynthetic activity by having more Chl *a* than Chl *b* at low and moderate seawater concentrations (Table 3), as shown in *V. negundo* by S. Das et al. (2024). Nevertheless, at high salinity (12 dS/m), *C. roseus* leaves lost 41% of their total Chl content, suggesting a major threat to the plant's survival.

With time and rising salinity, salt stress reduced the RWC of *C. roseus* compared to the control (Table 3). This decline could be the result of salt-induced restrictions on water accessibility and intake as well as damage to the root system of *C. roseus*. By building salt in the root zones and limiting water intake, salt stress lowers the root water potential and may result in osmotic imbalances (Kumar et al., 2021). According

to Bistgani et al. (2019), a drastic change in plant water status under saline conditions may be due to high concentrations of $Na^+$ uptake in plants and to delay the osmoregulation of the salinity tolerance threshold. These findings are consistent with other studies conducted on the plants *Prosopis alba* (Meloni et al., 2004), *Z. mays* (Çiçek & Çakirlar, 2002), and *Shepherdia argentea* (Qin et al., 2010).

## Biochemical Traits Caused by Seawater Salinity and Their Impact

### *Pro and Soluble Sugar Content*

In cellular metabolism, salt toxicity was avoided by organic osmolyte accumulation, which greatly assisted with osmotic adjustments, preserved turgidity, and prevented cellular metabolism from salt toxicity (S. Das et al., 2024). *C. roseus* accumulates greater Pro (Figure 2a), which may significantly play a role in osmotic adaptation and tolerance to salt as observed in different plant species (Azeem et al., 2019; S. Das et al., 2024; Sharif et al., 2018). The increased Pro could regulate the cytosolic pH buffer for subcellular structures, scavenge singlet oxygen and hydroxyl radicals, and lessen the acidification of cells caused by salinity stress (Isayenkov & Maathuis, 2019). Furthermore, the fact that elevated Pro concentrations build up in the cytosol in response to salinity without changing cellular structure or metabolism may be explained by the fact that Pro has a zwitterion property, which is known to be important in the osmoregulation of stressed tissues (Goyal & Asthir, 2010). It may facilitate the acclimation of *C. roseus* plants to salt stress. Comparable outcomes were found for several therapeutic plants, including moringa (Azeem et al., 2023), chamomile (Banerjee & Roychoudhury, 2017), basak (Obaidullah et al., 2022), and neem (Jahan et al., 2018).

The results of this study indicate that the accumulation of Pro in response to low (4 dS/m) and moderate (8 dS/m) salt concentrations may be a key component in salt tolerance. Nevertheless, under conditions of higher salinity (12 dS/m), hyperaccumulation of Pro was observed at 90 and 120 days in contrast to moderate salinity, indicating impaired growth performance and increased damage in response to salt stress. It has been suggested that the primary cause of the hyperaccumulation of Pro in leaf tissues following high salinity treatment may be leaf damage or a symptom of salt stress. Moreover, Pro builds up in leaves to preserve turgor and Chl concentrations, shielding plant photosynthesis from salt stress (Hnilickova et al., 2021).

Our findings also concur with Aldesuquy et al. (2012), who found that soil soaked in seawater greatly escalated the overall number of soluble sugars. Several researchers have also reported that starch and sugar buildup was exacerbated by salt stress (Sadak, 2019; Sen et al., 2022). Under salt stress, sugar buildup prevents structural and functional alterations of soluble proteins' membrane degradation (Sen et al., 2022).

### *Alternations of Oxidative Stress Indicators and Antioxidant Defense System under Seawater Stress*

Plants suffer oxidative damage when severe salt stress disrupts the electron transport path. The extra energy released during electrochemical reactions might produce more ROS (such as $H_2O_2$) through the Mehler reaction. Damage markers such as $H_2O_2$, EL, and MDA are associated with a series of free radical production events that can impact macromolecules and cellular structures. In the current study, *C. roseus* leaves' $H_2O_2$ and MDA levels significantly increased due to saltwater stress (Figures 4a and 4b). The MDA, $H_2O_2$ concentration, and EL in *C. roseus* increased more steeply with severe salinity (12 dS/m) and extended exposure time (120 days) in comparison to low and moderate salinities (4–8 dS/m). The findings indicate that *C. roseus* can protect membranes from salt-induced damage up to a particular salinity level (Figures 4a–4c). Increased $H_2O_2$ concentrations are directly associated with membrane and pigment deterioration, which turnover-reduces the photosynthetic apparatus and produces more radicals.

However, $H_2O_2$ also functions as a signaling molecule for stress tolerance (Mehmood et al., 2023; Saleem et al., 2022). $H_2O_2$ appears to have been used in low- and moderate-salinity states to identify and manage saltwater stress, which helped to activate antioxidant molecules (phenol and flavonoids) and high antioxidant capacity (DPPH, $ABTS^+$) (Table 4; Figure 4). Significantly intact membrane permeability (EL) of the plant, elevated quantities of stress-adaptive compounds (pro and soluble sugars), and minimal plant DW loss at this salinity all suggest that $H_2O_2$ has a favorable role in *C. roseus*'s ability to withstand salt at low and moderate salinities (4–8 dS/m). Elevated MDA and EL levels indicate that the cytotoxic levels of $Na^+$ caused ROS to surpass the plants' threshold limit, damaging membranes and cell structure when salt stress reached high salinity (12 dS/m). These findings align with earlier research conducted in *V. negundo* (S. Das et al., 2024) and *Salvadora persica* (Rangani et al., 2016). It could cause the plant's delayed growth and noticeable biomass loss in high-salt conditions.

When the plant underwent salinity stress, it activated both enzymatic and non-enzymatic antioxidants, which scavenged ROS and reduced oxidative stress and plant cell damage. According to the current study, *C. roseus* responded to salinity stress by increasing their APX and CAT activities (Figures 4d and 4e), as reported in other species such as *V. negundo* (S. Das et al., 2024), *Moringa oleifera* (Azeem et al., 2023), *Solanum lycopersicum* (Islam et al., 2023), *Oryza sativa* (Roy et al., 2019), *Brassica juncea* (Ahmad et al., 2015), and *Glycine max* (Weisany et al., 2012). APX normally uses ascorbate to convert $H_2O_2$ into water in the chloroplast, whereas CAT usually works in the cytoplasm. As ROS levels rose, antioxidant enzyme activity in the plant progressively increased (Apel & Hirt, 2004). At low and moderate salinities, increased CAT activity and stable APX

levels show a balanced regulation of ROS with little membrane damage (unaffected EL). *C. roseus* apparently regulates the amount of ROS in the cytoplasm and chloroplasts at a low energy cost. According to other data, $H_2O_2$ was generated in the chloroplast but remained in the cytoplasm due to altered APX but elevated CAT activity (Azeem et al., 2023).

However, the activities of CAT and APX significantly increase at higher salinities, indicating a major ROS burst across the cell and advocating for robust protection at the cytoplasmic and intracellular levels. As a result, managing oxidative stress was energy-intensive and significantly affected biomass and growth (Tables 1, 2, and 4; Figure 1). According to the current study, higher $H_2O_2$, MDA, and EL levels, as well as the strong correlation between those levels and higher antioxidant enzyme activity, are in line with the antioxidative responses of other salt-tolerant plants (Abogadallah, 2010; Subudhi & Baisakh, 2011).

*Catharanthus roseus* employs a well-differentiated defense mechanism consisting of antioxidant enzymes and powerful antioxidant chemical substances to protect the oxidative balance of cells. Total phenols and flavonoids are examples of antioxidant molecules that actively absorb free radicals; nevertheless, the quantity and composition of these molecules are strongly proportional to the degree of applied stress (de Abreu & Mazzafera, 2005). The levels of TPC and TFC ascended linearly with salinity (Table 4). Similarly, increasing salinity increased antioxidant capacity as measured by DPPH and $ABTS^+$, consistent with earlier research (Bekka et al., 2022). A lesser increase in antioxidant molecules and their activity is indicative of a regulatory response at low and moderate salinities. These molecules are notably more active and numerous, which eventually causes a steep drop in growth and implies a perceived need to respond rapidly to an oxidative burst in extremely high salinity. According to reports from *M. oleifera* and other Algerian medicinal plants (Djeridane et al., 2006; Meireles et al., 2020), these elements are significantly correlated with considerably greater radical scavenging (DPPH and $ABTS^+$) capacities. It suggests that these components play a major role in *C. roseus*'s antioxidant defense.

## Mineral Ion Homeostasis in Response to Seawater Stress

The effects of seawater stress on mineral absorption and transport are expected to substantially impact plants' capacity to survive salt (Taïbi et al., 2016). The ion analysis conducted for this study revealed that *C. roseus* plants' roots had accumulated more $Na^+$ than their leaves. However, their leaves had larger amounts of $K^+$ and $Ca^{2+}$ (Figures 5a, 5b, and 5c). This varied ion distribution in different organs leads to the greater $K^+/Na^+$ ratio in leaves compared to roots and the superior preferential transport capacities for $K^+$, $Ca^{2+}$, and $Mg^{2+}$ over $Na^+$ from roots to leaves with an increasing amount of salt (Figure 5a-5d). Higher concentrations of

$Na^+$ create physiological drought, which prevents plants from absorbing water and upsets the osmotic balance. According to Kronzucker et al. (2013), the accumulation of $Na^+$ in the cytosol hinders rubisco activity during the Calvin cycle, hence adversely affecting the photosynthetic process. It is generally agreed upon that increased root $Na^+$ ion concentrations relative to shoots were indicative of reduced $Na^+$ translocation to the shoots. As a result, the plants showed an improved tolerance to salt.

The results of the current study are well supported by data from studies on melons (Sivritepe et al., 2003) and maize (Turan et al., 2010). It is also important to keep in mind that $K^+$ is essential for plants to withstand salinity. It is necessary in high concentrations to reduce osmotic stress in a salinity-induced stress condition. According to Bistgani et al. (2019), salt stress promotes the transfer of $K^+$ to the xylem, preserving the water balance and ideal $K^+/Na^+$ ratio in the xylem. The concentrations of $Ca^{2+}$ and $Mg^{2+}$ in the leaves of the seawater-stressed *C. roseus* plants were significantly higher than in the roots (Figures 5c and 5d). This finding may indicate that ion exclusion techniques facilitate the absorption of other advantageous nutrients while decreasing the inhibitory effects of $Na^+$. Moreover, greater $Mg^{2+}$ concentrations in leaves may also aid in the production of Chls, the transportation of photoassimilates, and the preservation of the chloroplast ultrastructure, all essential for preserving the best possible photosynthesis in saline environments (Isayenkov & Maathuis, 2019).

Dendrogram and PCA biplot analysis clearly demonstrated that, at 8 dS/m seawater stress, physio-biochemical features, such as $H_2O_2$, MDA, Pro, SS, DPPH, TPhe, and $RNa^+$ were upregulated, whereas $LNa^+$, $ABTS^+$, EL, Tfla contents were negatively affected at 12 dS/m salinity (Figure 5).

## CONCLUSION

According to the findings, *C. roseus* could tolerate moderate salinity (8 dS/m) by managing its physio-biochemical traits, ion toxicity, and oxidative stress; nevertheless, biomass accumulation was impaired. However, even though this plant may produce higher concentrations of some biochemical features when cultivated in highly salinized soils (12 dS/m), it is unable to overlook the notable drop in dry biomass (72%). This research implies that *C. roseus*'s antioxidant defense system might allow it to withstand salt, making it appropriate for cultivation close to coastal regions with salinities of about 8 dS/m. It would enhance Bangladesh's economy and public health while also meeting the country's expanding demand for medicinal plants. Furthermore, land in a salinity-affected area that is not currently being farmed is appropriate for producing medicinal plants. Finally, on-farm studies should be accomplished in saline-prone areas under actual field conditions prior to it being economically cultivated in saline locations.

## ACKNOWLEDGMENTS

The authors greatly appreciate Bangabandhu Sheikh Mujibur Rahman Agricultural University's Research Management Wing, Bangladesh (RMW-GN 22) for providing funds and support for this experiment. They are also grateful for the assistance with their research from the Department of Agroforestry and Environment technical personnel at Bangabandhu Sheikh Mujibur Rahman Agricultural University in Bangladesh.

## REFERENCES

Abdelhamid, M. T., Rady, M. M., Osman, A. S., & Abdalla, M. A. (2013). Exogenous application of proline alleviates salt-induced oxidative stress in *Phaseolus vulgaris* L. plants. *The Journal of Horticultural Science and Biotechnology*, *88*(4), 439-446. https://doi.org/10.1080/14620316.2013.11512989

Abdul-Hafeez, E. Y., Karamova, N. S., & Ilinskaya, O. N. (2014). Antioxidant activity and total phenolic compound content of certain medicinal plants. *International Journal of Biosciences*, *5*(9), 213-222.

Abogadallah, G. M. (2010). Insights into the significance of antioxidative defense under salt stress. *Plant Signaling and Behavior*, *5*(4), 369-374. https://doi.org/10.4161/psb.5.4.10873

Acosta-Motos, J. R., Ortuno, M. F., Bernal-Vicente, A., Diaz-Vivancos, P., Sanchez-Blanco, M. J., & Hernandez, J. A. (2017). Plant responses to salt stress: Adaptive mechanisms. *Agronomy*, *7*(1), 18. https://doi.org/10.3390/agronomy7010018

Ahmad, P., Sarwat, M., Bhat, N. A., Wani, M. R., Kazi, A. G., & Tran, L.-S. P. (2015). Alleviation of cadmium toxicity in *Brassica juncea* L. (Czern. & Coss.) by calcium application involves various physiological and biochemical strategies. *PLOS One*, *10*(1), e0114571. https://doi.org/10.1371/journal.pone.0114571

Akter, R., Hasan, N., Reza, F., Asaduzzaman, M., Begum, K., & Shammi, M. (2023). Hydrobiology of saline agriculture ecosystem: A review of scenario change in south-west region of Bangladesh. *Hydrobiology*, *2*(1), 162-180. https://doi.org/10.3390/hydrobiology2010011

Alam, M. A., Juraimi, A. S., Rafii, M. Y., & Abdul Hamid, A. (2015). Effect of salinity on biomass yield and physiological and stem-root anatomical characteristics of purslane (*Portulaca oleracea* L.) accessions. *BioMed Research International*, *2015*, 105695. https://doi.org/10.1155/2015/105695

Aldesuquy, H. S., Baka, Z. A., El-Shehaby, O. A., & Ghanem, H. E. (2012). Efficacy of seawater salinity on osmotic adjustment and solutes allocation in wheat (*Triticum aestivum*) flag leaf during grain filling. *International Journal of Plant Physiology and Biochemistry*, *4*(3), 33-45. https://doi.org/10.5897/IJPPB11.059

Ali, S., Ullah, S., Khan, M. N., Khan, W. M., Razak, S. A., Wahab, S., Hafeez, A., Bangash, S. A. K., & Poczai, P. (2022). The effects of osmosis and thermo-priming on salinity stress tolerance in *Vigna radiata* L. *Sustainability*, *14*(19), 12924. https://doi.org/10.3390/su141912924

Apel, K., & Hirt, H. (2004). Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology*, *55*, 373-399. https://doi.org/10.1146/annurev.arplant.55.031903.141701

Azeem, M., Pirjan, K., Qasim, M., Mahmood, A., Javed, T., Muhammad, H., Yang, S., Dong, R., Ali, B., & Rahimi, M. (2023). Salinity stress improves antioxidant potential by modulating physio-biochemical responses in *Moringa oleifera* Lam. *Scientific Reports*, *13*, 2895. https://doi.org/10.1038/s41598-023-29954-6

Azeem, M., Qasim, M., Abbasi, M. W., Tayyab., Sultana, R., Adnan, M. Y., & Ali, H. (2019).

Salicylic acid seed priming modulates some biochemical parameters to improve germination and seedling growth of salt stressed wheat (*Triticum aestivum* L.). *Pakistan Journal of Botany*, *51*(2), 385-391. https://doi.org/10.30848/PJB2019-2(1)

Banerjee, A., & Roychoudhury, A. (2017). Effect of salinity stress on growth and physiology of medicinal plants. In M. Ghorbanpour & A. Varma (Eds.), *Medicinal plants and environmental challenges* (pp. 177-188). Springer. https://doi.org/10.1007/978-3-319-68717-9_10

Bates, L. S., Waldren, R. P., & Teare, I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil*, *39*, 205-207. https://doi.org/10.1007/BF00018060

Bekka, S., Tayeb-Hammani, K., Boucekkine, I., El-Amin Aissiou, M. Y., & Djazouli, Z. E. (2022). Adaptation strategies of *Moringa oleifera* under drought and salinity stresses. *Ukrainian Journal of Ecology*, *12*(4), 8-16.

Bistgani, Z. E., Hashemi, M., DaCosta, M., Craker, L., Maggi, F., & Morshedloo, M. R. (2019). Effect of salinity stress on the physiological characteristics, phenolic compounds and antioxidant activity of *Thymus vulgaris* L. and *Thymus daenensis* Celak. *Industrial Crops and Products*, *135*, 311-320. https://doi.org/10.1016/J.INDCROP.2019.04.055

Cai, Y., Luo, Q., Sun, M., & Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences*, *74*(17), 2157-2184. https://doi.org/10.1016/j.lfs.2003.09.047

Cassaniti, C., Romano, D., & Flowers, T. J. (2012). The response of ornamental plants to saline irrigation water. In I. García-Garizábal & R. Abrahao (Eds.), *Irrigation - Water management, pollution and alternative strategies* (pp.131-158). InTech. https://doi.org/10.5772/31787

Chen, M. (2014). Chlorophyll modifications and their spectral extension in oxygenic photosynthesis. *Annual Review of Biochemistry*, *83*, 317-340. https://doi.org/10.1146/annurev-biochem-072711-162943

Çiçek, N., & Çakirlar, H. (2002). The effect of salinity on some physiological parameters in two maize cultivars. *Bulgarian Journal of Plant Physiology*, *28*(1-2), 66-74.

Das, A. K., Anik, T. R., Rahman, M. M., Keya, S. S., Islam, M. R., Rahman, M. A., Sultana, S., Ghosh, P. K., Khan, S., Ahamed, T., Ghosh, T. K., Tran, L. S.-P., & Mostofa, M. G. (2022). Ethanol treatment enhances physiological and biochemical responses to mitigate saline toxicity in soybean. *Plants*, *11*(3), 272. https://doi.org/10.3390/plants11030272

Das, S., Parvin, S., Islam, M. M., Rahman, A., Mohi-Ud-Din, M., Ahmed, M., Miah, M. G., Alamri, S., & ALMunqedhi, B. M. A. (2024). Morpho-physiological and biochemical responses of *Vitex negundo* to seawater induced salt stress. *South African Journal of Botany*, *166*, 648-662. https://doi.org/10.1016/j.sajb.2024.01.069

Das, S., & Sharangi, A. B. (2017). Madagascar periwinkle (*Catharanthus roseus* L.): Diverse medicinal and therapeutic benefits to humankind. *Journal of Pharmacognosy and Phytochemistry*, *6*(5), 1695-1701.

Dasgupta, S., Hossain, M. M., Huq, M., & Wheeler, D. (2015). Climate change and soil salinity: The case of coastal Bangladesh. *Ambio*, *44*, 815-826. https://doi.org/10.1007/s13280-015-0681-5

Dawood, M. G., Taie, H. A. A., Nassar, R. M. A., Abdelhamid, M. T., & Schmidhalter, U. (2014). The changes induced in the physiological, biochemical and anatomical characteristics of *Vicia faba* by the exogenous application of proline under seawater stress. *South African Journal of Botany*, *93*, 54-63. https://doi.org/10.1016/j.sajb.2014.03.002

de Abreu, I. N., & Mazzafera, P. (2005). Effect of water and temperature stress on the content of active constituents of *Hypericum brasiliense* Choisy. *Plant Physiology and Biochemistry*, *43*(3), 241-248. https://doi.org/10.1016/j.plaphy.2005.01.020

Djeridane, A., Yousfi, M., Nadjemi, B., Boutassouna, D., Stocker, P., & Vidal, N. (2006). Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. *Food Chemistry*, *97*(4), 654-660. https://doi.org/10.1016/j.foodchem.2005.04.028

Farooq, T. H., Rafay, M., Basit, H., Shakoor, A., Shabbir, R., Riaz, M. U., Ali, B., Kumar, U., Qureshi, K. A., & Jaremko, M. (2022). Morpho-physiological growth performance and phytoremediation capabilities of selected xerophyte grass species toward Cr and Pb stress. *Frontiers in Plant Science*, *13*, 997120. https://doi.org/10.3389/fpls.2022.997120

Geissler, N., Hussin, S., & Koyro, H.-W. (2009). Interactive effects of NaCl salinity and elevated atmospheric $CO_2$ concentration on growth, photosynthesis, water relations and chemical composition of the potential cash crop halophyte *Aster tripolium* L. *Environmental and Experimental Botany*, *65*(2-3), 220-231. https://doi.org/10.1016/j.envexpbot.2008.11.001

Gengmao, Z., Quanmei, S., Yu, H., Shihui, L., & Changhai, W. (2014). The physiological and biochemical responses of a medicinal plant (*Salvia miltiorrhiza* L.) to stress caused by various concentrations of NaCl. *PLOS One*, *9*(2), e89624. https://doi.org/10.1371/journal.pone.0089624

Goyal, M., & Asthir, B. (2010). Polyamine catabolism influences antioxidative defense mechanism in shoots and roots of five wheat genotypes under high temperature stress. *Plant Growth Regulation*, *60*, 13-25. https://doi.org/10.1007/s10725-009-9414-8

Gururani, M. A., Venkatesh, J., & Tran, L. S. P. (2015). Regulation of photosynthesis during abiotic stress-induced photoinhibition. *Molecular Plant*, *8*(9), 1304-1320. https://doi.org/10.1016/j.molp.2015.05.005

Haddadi, B. S., Hassanpour, H., & Niknam, V. (2016). Effect of salinity and waterlogging on growth, anatomical and antioxidative responses in *Mentha aquatica* L. *Acta Physiologiae Plantarum*, *38*, 119. https://doi.org/10.1007/s11738-016-2137-3

Hand, M. J., Taffouo, V. D., Nouck, A. E., Nyemene, K. P. J., Tonfack, B., Meguekam, T. L., & Youmbi, E. (2017). Effects of salt stress on plant growth, nutrient partitioning, chlorophyll content, leaf relative water content, accumulation of osmolytes and antioxidant compounds in pepper (*Capsicum annuum* L.) cultivars. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, *45*(2), 481-490. https://doi.org/10.15835/nbha45210928

Hassanein, R. A., Hassanein, A. A., Haider, A. S., & Hashem, H. A. (2009). Improving salt tolerance of *Zea mays* L. plants by presoaking their grains in glycine betaine. *Australian Journal of Basic and Applied Sciences*, *3*(2), 928-942.

Hnilickova, H., Kraus, K., Vachova, P., & Hnilicka, F. (2021). Salinity stress affects photosynthesis, malondialdehyde formation, and proline content in *Portulaca oleracea* L. *Plants*, *10*(5), 845. https://doi.org/10.3390/plants10050845

Isayenkov, S. V., & Maathuis, F. J. M. (2019). Plant salinity stress: Many unanswered questions remain. *Frontiers in Plant Science*, *10*, 80. https://doi.org/10.3389/fpls.2019.00080

Islam, M. M., Jahan, K., Sen, A., Urmi, T. A., Haque, M. M., Ali, H. M., Siddiqui, M. H., & Murata, Y. (2023). Exogenous application of calcium ameliorates salinity stress tolerance of tomato (*Solanum lycopersicum* L.) and enhances fruit quality. *Antioxidants*, *12*(3), 558. https://doi.org/10.3390/antiox12030558

Jahan, I., Parvin, S., Miah, M. G., & Ahmed, J. U. (2018). Effect of salinity on the physiological and biochemical responses of neem. *International Journal of Environmental and Agriculture Research*, *4*(5), 47-54.

Jaleel, C. A., Lakshmanan, G. M. A., Gomathinayagam, M., & Panneerselvam, R. (2008). Triadimefon induced salt stress tolerance in *Withania somnifera* and its relationship to antioxidant defense system. *South African Journal of Botany*, *74*(1), 126-132. https://doi.org/10.1016%2Fj.sajb.2007.10.003

Kronzucker, H. J., Coskun, D., Schulze, L. M., Wong, J. R., & Britto, D. T. (2013). Sodium as nutrient and toxicant. *Plant and Soil*, *369*, 1-23. https://doi.org/10.1007/s11104-013-1801-2

Kumar, S., Li, G., Yang, J., Huang, X., Ji, Q., Liu, Z., Ke, W., & Hou, H. (2021). Effect of salt stress on growth, physiological parameters, and ionic concentration of water dropwort (*Oenanthe javanica*) cultivars. *Frontiers in Plant Science*, *12*, 660409. https://doi.org/10.3389/fpls.2021.6604092021

Li, Z., Geng, W., Tan, M., Ling, Y., Zhang, Y., Zhang, L., & Peng, Y. (2022). Differential responses to salt stress in four white clover genotypes associated with root growth, endogenous polyamines metabolism, and sodium/potassium accumulation and transport. *Frontiers in Plant Science*, *13*, 896436. https://doi.org/10.3389/fpls.2022.896436

Lutts, S., Kinet, J. M., & Bouharmont, J. (1996). NaCl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. *Annals of Botany*, *78*(3), 389-398. https://doi.org/10.1006/anbo.1996.0134

Mafakheri, A., Siosemardeh, A., Bahramnejad, B., Struik, P. C., & Sohrabi, Y. (2010). Effect of drought stress on yield, proline and chlorophyll contents in three chickpea cultivars. *Australian Journal of Crop Science*, *4*(8), 580-585.

Mehmood, S., Khatoon, Z., Amna., Ahmad, I., Muneer, M. A., Kamran, M. A., Ali, J., Ali, B., Chaudhary, H. J., & Munis, M. F. H. (2023). *Bacillus* sp. PM31 harboring various plant growth-promoting activities regulates *Fusarium* dry rot and wilt tolerance in potato. *Archives of Agronomy and Soil Science*, *69*(2), 197-211. https://doi.org/10.1080/03650340.2021.1971654

Meireles, D., Gomes, J., Lopes, L., Hinzmann, M., & Machado, J. (2020). A review of properties, nutritional and pharmaceutical applications of *Moringa oleifera*: Integrative approach on conventional and traditional Asian medicine. *Advances in Traditional Medicine*, *20*, 495-515. https://doi.org/10.1007/s13596-020-00468-0

Meloni, D. A., Gulotta, M. R., Martínez, C. A., & Oliva, M. A. (2004). The effects of salt stress on growth, nitrate reduction and proline and glycinebetaine accumulation in *Prosopis alba*. *Brazilian Journal of Plant Physiology*, *16*(1), 39-46. https://doi.org/10.1590/S1677-04202004000100006

Mostofa, M. G., Saegusa, D., Fujita, M., & Tran, L.-S. P. (2015). Hydrogen sulfide regulates salt tolerance in rice by maintaining $Na^+/K^+$ balance, mineral homeostasis and oxidative metabolism under excessive salt stress. *Frontiers in Plant Science*, *6*, 1055. https://doi.org/10.3389/fpls.2015.01055

Munns, R., & Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, *59*, 651-681. https://doi.org/10.1146/annurev.arplant.59.032607.092911

Nakano, Y., & Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and Cell Physiology*, *22*(5), 867-880. https://doi.org/10.1093/oxfordjournals.pcp.a076232

Noctor, G., Mhamdi, A., & Foyer, C. H. (2016). Oxidative stress and antioxidative systems: Recipes for successful data collection and interpretation. *Plant, Cell and*

*Environment*, *39*(5), 1140-1160. https://doi.org/10.1111/pce.12726

Obaidullah, A. J. M., Parvin, S., Saha, S. R., Biswas, M. S., Das, S., Ahmed, T., & Sultana, S. (2022). Salinity stress effects on some morpho-physiological and biochemical traits of Basak. *European Journal of Agriculture and Food Sciences*, *4*(3), 50-56. https://doi.org/10.24018/ejfood.2022.4.3.496

Pan, Y.-Q., Guo, H., Wang, S.-M., Zhao, B., Zhang, J.-L., Ma, Q., Yin, H.-J., & Bao, A.-K. (2016). The photosynthesis, $Na^+/K^+$ homeostasis and osmotic adjustment of *Atriplex canescens* in response to salinity. *Frontiers in Plant Science*, *7*, 848. https://doi.org/10.3389/fpls.2016.00848

Qin, J., Dong, W. Y., He, K. N., Yu, Y., Tan, G. D., Han, L., Dong, M., Zhang, Y. Y., Zhang, D., Li, A. Z., & Wang, Z. L. (2010). NaCl salinity-induced changes in water status, ion contents and photosynthetic properties of *Shepherdia argentea* (Pursh) Nutt. seedlings. *Plant, Soil and Environment*, *56*(7), 325-332. https://doi.org/10.17221/209/2009-PSE

Rahman, M. M., Rahman, M. A., Miah, M. G., Saha, S. R., Karim, M. A., & Mostofa, M. G. (2017). Mechanistic insight into salt tolerance of *Acacia auriculiformis*: The importance of ion selectivity, osmoprotection, tissue tolerance, and $Na^+$ exclusion. *Frontiers in Plant Science*, *8*, 155. https://doi.org/10.3389/fpls.2017.00155

Rahneshan, Z., Nasibi, F., & Moghadam, A. A. (2018). Effects of salinity stress on some growth, physiological, biochemical parameters and nutrients in two pistachio (*Pistacia vera* L.) rootstocks. *Journal of Plant Interactions*, *13*(1), 73-82. https://doi.org/10.1080/17429145.2018.1424355

Rangani, J., Parida, A. K., Panda, A., & Kumari, A. (2016). Coordinated changes in antioxidative enzymes protect the photosynthetic machinery from salinity induced oxidative damage and confer salt tolerance in an extreme halophyte *Salvadora persica* L. *Frontiers in Plant Science*, *7*, 50. https://doi.org/10.3389/fpls.2016.00050

Roy, P. R., Tahjib-Ul-Arif, M., Polash, M. A. S., Hossen, M. Z., & Hossain, M. A. (2019). Physiological mechanisms of exogenous calcium on alleviating salinity-induced stress in rice (*Oryza sativa* L.). *Physiology and Molecular Biology of Plants*, *25*, 611-624. https://doi.org/10.1007/s12298-019-00654-8

Sadak, M. S. (2019). Physiological role of trehalose on enhancing salinity tolerance of wheat plant. *Bulletin of the National Research Centre*, *43*, 53.

Sairam, R. K., Deshmukh, P. S., & Shukla, D. S. (1997). Tolerance of drought and temperature stress in relation to increased antioxidant enzyme activity in wheat. *Journal of Agronomy and Crop Science*, *178*(3), 171-178. https://doi.org/10.1111/j.1439-037X.1997.tb00486.x

Saleem, A., Zulfiqar, A., Ali, B., Naseeb, M. A., Almasaudi, A. S., & Harakeh, S. (2022). Iron sulfate ($FeSO_4$) improved physiological attributes and antioxidant capacity by reducing oxidative stress of *Oryza sativa* L. cultivars in alkaline soil. *Sustainability*, *14*(24), 16845. https://doi.org/10.3390/su142416845

Sarker, U., Islam, M. T., & Oba, S. (2018). Salinity stress accelerates nutrients, dietary fiber, minerals, phytochemicals and antioxidant activity in *Amaranthus tricolor* leaves. *PLOS One*, *13*(11), e0206388. https://doi.org/10.1371/journal.pone.0206388

Sen, A., Islam, M. M., Zaman, E., Ghosh, U. K., Momtaz, M. B., Islam, M. A., Urmi, T. A., Mamun, M. A. A., Rahman, M. M., Kamal, M. Z. U., Rahman, G. K. M. M., Haque, M. M., & Murata, Y. (2022). Agro-morphological, yield and biochemical responses of selected wheat (*Triticum aestivum* L.) genotypes to salt stress. *Agronomy*, *12*(12), 3027. https://doi.org/10.3390/agronomy12123027

Sharif, P., Seyedsalehi, M., Paladino, O., Van Damme, P., Sillanpää, M., & Sharifi, A. A.

(2018). Effect of drought and salinity stresses on morphological and physiological characteristics of canola. *International Journal of Environmental Science and Technology*, *15*, 1859-1866. https://doi.org/10.1007/s13762-017-1508-7

Sivritepe, N., Sivritepe, H. O., & Eris, A. (2003). The effects of NaCl priming on salt tolerance in melon seedlings grown under saline conditions. *Scientia Horticulturae*, *97*(3-4), 229-237. https://doi.org/10.1016/S0304-4238(02)00198-X

Subudhi, P. K., & Baisakh, N. (2011). *Spartina alterniflora* Loisel., a halophyte grass model to dissect salt stress tolerance. *In Vitro Cellular and Developmental Biology - Plant*, *47*, 441-457. https://doi.org/10.1007/s11627-011-9361-8

Sun, Y. L., Li, F., Su, N., Sun, X. L., Zhao, S. J., & Meng, Q. W. (2010). The increase in unsaturation of fatty acids of phosphatidylglycerol in thylakoid membrane enhanced salt tolerance in tomato. *Photosynthetica*, *48*, 400-408. https://doi.org/10.1007/s11099-010-0052-1

Taïbi, K., Taïbi, F., Abderrahim, L. A., Ennajah, A., Belkhodja, M., & Mulet, J. M. (2016). Effect of salt stress on growth, chlorophyll content, lipid peroxidation and antioxidant defence systems in *Phaseolus vulgaris* L. *South African Journal of Botany*, *105*, 306-312. https://doi.org/10.1016/j.sajb.2016.03.011

Tamanna, T., Islam, M. M., Chaity, A. R., Shams, S.-N.-U., Rasel, M. A., Haque, M. M., Miah, M. G., Alamri, S., & Murata, Y. (2023). Water relation, gas exchange characteristics and yield performance of selected mungbean genotypes under low soil moisture condition. *Agronomy*, *13*(4), 1068. https://doi.org/10.3390/agronomy13041068

Turan, M. A., Elkarim, A. H. A., Taban, N., & Taban, S. (2010). Effect of salt stress on growth and ion distribution and accumulation in shoot and root of maize plant. *African Journal of Agricultural Research*, *5*(7), 584-588.

Uematsu, K., Suzuki, N., Iwamae, T., Inui, M., & Yukawa, H. (2012). Increased fructose 1, 6-bisphosphate aldolase in plastids enhances growth and photosynthesis of tobacco plants. *Journal of Experimental Botany*, *63*(8), 3001-3009. https://doi.org/10.1093/jxb/ers004

Urmi, T. A., Islam, M. M., Zumur, K. N., Abedin, M. A., Haque, M. M., Siddiqui, M. H., Murata, Y., & Hoque, M. A. (2023). Combined effect of salicylic acid and proline mitigates drought stress in rice (*Oryza sativa* L.) through the modulation of physiological attributes and antioxidant enzymes. *Antioxidants*, *12*(7), 1438. https://doi.org/10.3390/antiox12071438

Wang, D., Gao, Y., Sun, S., Lu, X., Li, Q., Li, L., Wang, K., & Liu, J. (2022). Effects of salt stress on the antioxidant activity and malondialdehyde, solution protein, proline, and chlorophyll contents of three *Malus* species. *Life*, *12*(11), 1929. https://doi.org/10.3390/life12111929

Weisany, W., Sohrabi, Y., Heidari, G., Siosemardeh, A., & Ghassemi-Golezani, K. (2012). Changes in antioxidant enzymes activity and plant performance by salinity stress and zinc application in soybean (*Glycine max* L.). *Plant Omics*, *5*(2), 60-67.

Witham, F. H., Blaydes, D. F., & Devlin, R. M. (1971). Chlorophyll absorption spectrum and quantitative determination. *Experiments in Plant Physiology*, *1971*, 167-2.

Yemm, E. W., & Willis, A. J. (1954). The estimation of carbohydrates in plant extracts by anthrone. *Biochemical Journal*, *57*(3), 508-514. https://doi.org/10.1042/bj0570508

Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry*, *64*(4), 555-559. https://doi.org/10.1016/S0308-8146(98)00102-2

Pertanika J. Trop. Agric. Sci. 47 (4): 1291 - 1308 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Effects of Fresh and Composted *Azolla* on Soil Chemical Properties

**Nur Syahirah Abdul Rashid[1], Mohamadu Boyie Jalloh[1], Elisa Azura Azman[2], Azwan Awang[1], Osumanu Haruna Ahmed[3] and Nor Elliza Tajidin[2,4]***

[1]*Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, UMS Sandakan Branch, Sungai Batang Road, Mile 10, 90000 Sandakan, Sabah, Malaysia*

[2]*Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

[3]*Faculty of Agriculture, Sinaut Campus, Universiti Islam Sultan Sharif Ali, Km 33 Jln Tutong Kampong Sinaut, Tutong TB1741, Brunei Darussalam*

[4]*Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

## ABSTRACT

The rise in chemical fertilizer use in Malaysia raises concerns about soil degradation and potential long-term yield reductions, highlighting the importance of using organic matter for soil restoration. *Azolla* has been extensively studied as an alternative soil amendment due to its high nitrogen and nutrient content, as well as its rapid growth. However, the effects of fresh and composted *Azolla* amendments on soil chemical properties are not yet fully understood. A soil incubation study was thus conducted to determine the effects of fresh and composted *Azolla* on soil chemical properties over a 3-month incubation period. The soil treatments consisted of non-amended soil (control); fresh *Azolla* at 3, 6, and 9% w/w; and composted *Azolla* at 1, 2, and 3% w/w, with soil water holding capacity maintained at 55% throughout the incubation period. The collected soil samples were analyzed for soil pH and electrical conductivity (EC), total carbon (C) and nitrogen (N), available phosphorus, exchangeable bases—potassium (K), calcium, and magnesium, using inductively coupled plasma optical emission spectrometry, and cation exchange capacity (CEC). All data were subjected to variance analysis for statistical analysis. The study revealed significant effects of interaction between soil treatments and incubation periods for all soil parameters. At the end of the incubation period, the soil treated with 3% composted *Azolla* exhibited higher soil EC, total C and N, exchangeable K, and CEC compared to

ARTICLE INFO

*Article history:*
Received: 28 February 2024
Accepted: 14 May 2024
Published: 15 November 2024

DOI: https://doi.org/10.47836/pjtas.47.4.14

*E-mail addresses*:
nursyahirahrashid96@gmail.com (Nur Syahirah Abdul Rashid)
mbjalloh@ums.edu.my (Mohamadu Boyie Jalloh)
elisa@upm.edu.my (Elisa Azura Azman)
azwang@ums.edu.my (Azwan Awang)
ahmed.haruna@unissa.edu.bn (Osumanu Haruna Ahmed)
elliza.tajidin@upm.edu.my (Nor Elliza Tajidin)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

other soil treatments. The 3% fresh *Azolla* treatments were also observed to improve the soil's exchangeable calcium by the end of the incubation period. In conclusion, 3% composted *Azolla* is best to help restore soil nutrient levels for crop uptake.

*Keywords*: *Azolla microphylla*, clay soil, soil amendment, soil incubation, soil nutrients

## INTRODUCTION

Soil is a crucial natural resource that is vital for plant growth. It serves as a growth medium, supporting the plant's root system while simultaneously providing essential nutrients and moisture (Laruna et al., 2020). However, increasing soil degradation and agricultural waste outputs have become serious global challenges. Commonly, repetitive and unbalanced fertilizer applications promote organic matter mineralization and lead to a decline in overall soil fertility, such as decreased soil carbon reserves and increased soil acidity (B. Singh, 2018; Karam et al., 2021). Increasing environmental concerns related to the excessive use of chemical fertilizers highlight the need for thorough research into potential sustainable methods to address the associated risks.

Various soil amendments, including mineral, organic, and synthetic, are employed to improve soil fertility for crop growth. Long-term application of soil amendments improves many soil variables, including soil texture, organic C, nutrient availability, crop growth and its environment, and microbes that are useful for crop production, compared to chemical fertilizers (V. K. Singh et al., 2022). Organic amendments, such as compost or green manure, are common amendments for boosting soil performance and crop productivity, where these amendments have low production costs (Trupiano et al., 2017; V. K. Singh et al., 2022). For instance, amendments on clay soil can be advantageous as clay's greater surface area and chemical bonding capacity make it an ideal site for the formation of macro- and micro-aggregates, preserving the organic matter (Bronick & Lal, 2005; Ge et al., 2019; Oades, 1988). Hence, the increasing production of eco-friendly amendments has made *Azolla* a viable option for enhancing clayey soil properties (Marzouk et al., 2023).

Farmers in certain limited areas of China and Vietnam have been using *Azolla* for centuries. These countries began conducting research to expand the utilization of *Azolla* in crop production as far back as the early sixties (van Hove & Lejeune, 1996). Indonesia has also studied the *Azolla* amendment extensively due to its high nitrogen (N) content and rapid growth (Setiawati, Damayani, et al., 2018; Widiastuti et al., 2018). In Asia, it is commonly grown as an intercrop in lowland paddy fields or as a pre-season crop before planting (Thapa & Poudel, 2021). Further, according to the Malaysian Agricultural Research and Development Institute (MARDI), local farmers have integrated *Azolla* into paddy fields (Shafiee et al., 2021). However, the utilization of the *Azolla* amendment in Malaysia, particularly

in the state of Sabah on Borneo Island in East Malaysia, remains an area that has not yet been thoroughly studied.

Despite its apparent beneficial qualities, *Azolla* is considered one of Europe's most harmful invasive aquatic plants (Pinero-Rodríguez et al., 2021). Djojosuwito (2000) stated that spreading about 500 kg of *Azolla* seed per ha in a paddy field led to *Azolla* biomass increasing to 20,000 kg/ha within 2 weeks, indicating its rapid multiplication potential. Regular harvesting of the *Azolla* biomass for conversion into alternative soil amendments, such as compost, can help prevent the formation of such dense mats in paddy fields or other areas where it is cultivated. Interestingly, *Azolla* can be used in several forms, such as extracts, compost, green manure, and biochar.

Generally, in a day, *Azolla* fixes 75 mg N/g per dry weight and yields fresh weight of approximately 347 tonnes/ha in a year with about 868 kg N content, which is equivalent to 1,900 kg of urea (Yadav et al., 2014). *Azolla* is reported to be able to supply approximately 35–50% of fixed N to paddy fields, rendering it a perfect organic fertilizer for other crops as well, such as leafy vegetables, either in fresh or composted form (Pereira, 2018; Setiawati, Damayani, et al., 2018). Further, the study by Lestari et al. (2019) demonstrated that applying *Azolla* compost increased mustard green yield by improving the soil properties. The introduction of *Azolla* into the soil also improves other soil chemical properties such as CEC, exchangeable bases, and acidity, hence efficiently improving the nutrient uptake by crops (Barus et al., 2018; Sanjay-Swami & Singh, 2019b; Setiawati, Suryatmana, et al., 2018). Hence, *Azolla* is a viable alternative to reduce reliance on synthetic fertilizers in agriculture.

While the agronomic potential of using *the Azolla* amendment has been widely proposed, the effects of a single application of the *Azolla* amendment (i.e., fresh and composted *Azolla*) alone on soil properties over time have yet to be thoroughly studied. Thus, this study was conducted to determine the effects of fresh and composted *Azolla* on soil chemical properties over a three-month incubation period.

## MATERIALS AND METHODS

### Composted *Azolla* Preparation

A soil incubation study under field conditions was conducted in an insect-proof net house at the Faculty of Sustainable Agriculture, Universiti Malaysia Sabah. Fresh *Azolla* harvested from the insect-proof net house was cleaned and washed before being sun-dried for a few days prior to composting. Composted *Azolla* was prepared according to the method suggested by Jumadi et al. (2014), with some modifications to fit the study conditions. For the compost-making, a mixture of dried and fresh *Azolla* biomass and molasses was used at 9:6:2 of total weight, respectively. The mixture was placed in a black bin with dimensions of 40 cm diameter and 50 cm height (45 L). Water was added throughout the composting process to maintain the moisture content at 50–60% of the total weight. The composting process spanned 14 days, with the compost

undergoing the mesophilic stage for 3 days, the thermophilic stage for 5 days, and the curing phase for 6 days (Figure 1).

The matured compost was ready for harvest once the temperature was found to remain constant even after turning (30–34°C), the color changed to dark brown, easily crumbled, the pH was almost neutral, and EC was below 4 dS/m (Bernai et al., 1998; El-mrini et al., 2022; Kalamdhad & Kazmi, 2009; Khalib et al., 2020). The fresh and composted *Azolla* were characterized by pH, EC, C, N, C/N ratio, phosphorus (P), potassium (K), calcium (Ca), and magnesium (Mg) content before applying it to the soil. The pH and EC readings were taken with a pH/EC meter (PC 2700, Eutech Instruments, Singapore). The C and N of the *Azolla* biomass were determined with a CHN elemental analyzer (FP628, LECO Corporation, USA). Nutrient content (P, K, Ca, and Mg) of the fresh and composted *Azolla* was determined using the dry ashing method suggested by the AOAC International (2002), where the filtrate was analyzed using an inductively coupled plasma optical emission spectrometry (ICP-OES) instrument (Optima 2000DV, Perkin Elmer, USA).

### Soil Incubation: Experimental Design, Treatments, Laboratory Tests, and Statistical Analysis

This completely randomized design soil incubation study was carried out using plastic containers (20 cm diameter × 9.5 cm height), each filled with 2.5 kg of Typic Paleudults soil (Silabukan series). *Azolla* was applied to the soil as an amendment in the form of fresh biomass or compost at different rates at the beginning of the study, as shown in Table 1. Each soil treatment was replicated five times. At the beginning of the experiment, about 500 ml of deionized water was added to the soil. The soil's water-holding capacity was maintained at 55% using a soil sensor reader (WaterScout SMEC 300, Spectrum Technologies Inc.,

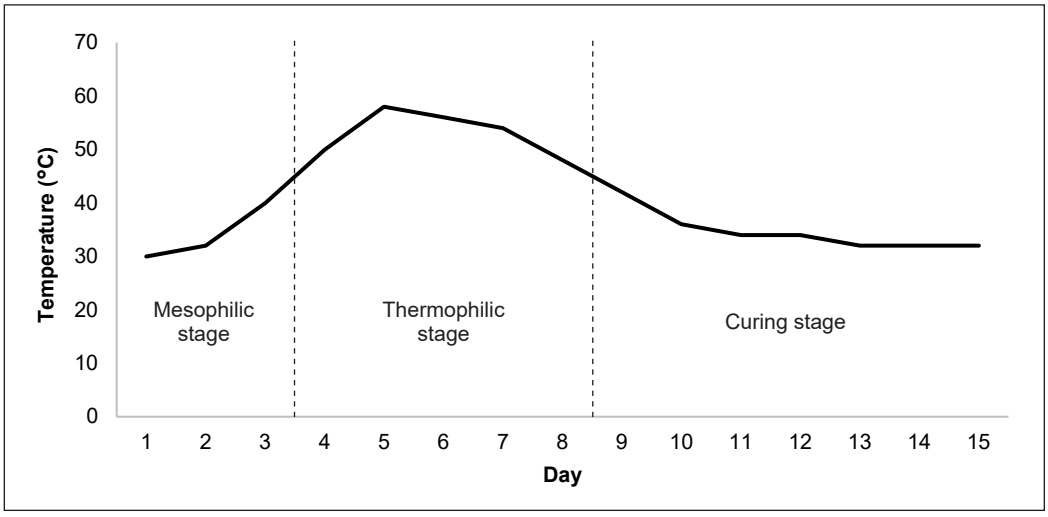



*Figure 1*. Temperature variation over a composting period

USA) throughout the incubation period by adding deionized water (Jumadi et al., 2014). Soil samples were collected at the beginning of the experiment and every one-month interval for up to three months. At the beginning of the study, the soil texture was determined using the hydrometer method suggested by D. Sarkar and Haldar (2010). The percentage of silt, clay, and sand was calculated, and the results were used to determine the textural class of the soil using the International Society of Soil Science (ISSS) textural triangle. The soil samples were analyzed for pH, EC, total C and N, available P, and exchangeable bases — K, Ca, Mg, and CEC. The pH and EC readings were measured using a pH/EC meter (PC 2700, Eutech Instruments, Singapore). The total C and N were determined using a CHN elemental analyzer (FP628, LECO Corporation, USA); soil available P was determined by mixing with concentrated hydrochloric acid (HCl) (Systerm, Malaysia) and sulphuric acid (Systerm, Malaysia) (Bray & Kurtz, 1945); then, the soil filtrate was mixed with Reagent B and analyzed by UV-Vis spectrophotometer (Genesys 10S, Thermo Scientific, USA) at 882 nanometer.

The soil exchangeable bases were determined by extracting the soil with 1 N ammonium acetate (Merck, Germany) (Soil Survey Staff, 2014). The extraction was then measured for the exchangeable bases using an ICP-OES instrument. The procedures for soil exchangeable bases determination were continued to determine the soil CEC. Ethanol (Systerm, Malaysia) was added to a leaching tube with 0.05 M potassium sulfate (Merck, Germany) before extracting the soil. The resulting extraction was mixed with sodium hydroxide (Merck, Germany) in a distillation glass, with boric acid (Merck, Germany) used as an indicator in a conical flask. The distillation process was carried out using a unit (K350, Büchi, Switzerland), lasting 4 min (with a color change from purple to green). The green solution was then titrated with 0.01 N HCl for neutralization, changing the color to purple. The amount of HCl used was recorded. The Statistical Analysis System (SAS) software (version 9.4) was used for all data analysis. Where significant interaction effects between the factors were observed, the simple effects of the incubation period on the measured variables were determined for each soil treatment using analysis of variance (ANOVA) and mean comparison by the least significant difference test (LSD) at a 95% confidence level.

## RESULTS

There were significant differences between fresh and composted *Azolla* for the pH, EC, C, C/N, P, K, and Ca content but not for the N and Mg content (Table 2). Due to the addition of molasses, the composted *Azolla* had higher P, K, and Ca contents than the fresh *Azolla*. The soil texture was typical clay (clay: 74.96%, silt: 21.04%, sand: 4.00%). The initial soil pH was 6.93, placed within the slightly acidic to neutral range, with an EC of 0.12 dS/m. Meanwhile, the total C and N content were 2.75 and 0.66%, respectively. The soil contained 6.23 mg/kg available P, with exchangeable K, Ca, and

Mg values of 1.04, 9.31, and 9.22 cmolc/kg, respectively. Further, the initial CEC of the soil was reported to be 20.52 cmolc/kg.

The soil amendment (SA) and incubation period (IP) affected the soil parameters significantly, as shown in Table 3.

Table 1
Azolla *amendment treatments on soil*

| Treatment | Rate of application (%) | Weight of *Azolla* amendment (g/container) |
|---|---|---|
| Non-amended (control) | 0 | 0 |
| Fresh *Azolla* | 3 | 75 |
| Fresh *Azolla* | 6 | 150 |
| Fresh *Azolla* | 9 | 225 |
| Composted *Azolla* | 1 | 25 |
| Composted *Azolla* | 2 | 50 |
| Composted *Azolla* | 3 | 75 |

Table 2
*Chemical properties of fresh and composted* Azolla

| Parameter | Fresh *Azolla* | Composted *Azolla* | t-test |
|---|---|---|---|
| pH | 6.75 | 7.67 | *** |
| EC (dS/m) | 3.31 | 3.02 | ** |
| C (%) | 38.34 | 35.35 | *** |
| N (%) | 4.28 | 4.48 | ns |
| C/N ratio | 9.19 | 7.92 | * |
| P (mg/L) | 44.08 | 68.20 | * |
| K (mg/L) | 366.60 | 468.70 | *** |
| Ca (mg/L) | 110.70 | 171.20 | ** |
| Mg (mg/L) | 69.65 | 76.32 | ns |

*Note*. * Significant at $P \leq 0.05$ probability level; ** Significant at $P \leq 0.01$ probability level; *** Significant at $P \leq 0.001$; ns = Not significant

Table 3
*Summary of main and interaction effects of soil amendments on soil properties throughout the incubation period*

| Factor | pH | EC (dS/m) | Total (%) | | Available P (mg/kg) | Exchangeable (cmolc/kg) | | | CEC (cmolc/kg) |
|---|---|---|---|---|---|---|---|---|---|
| | | | C | N | | K | Ca | Mg | |
| Soil amendment | *** | *** | *** | *** | *** | *** | *** | *** | *** |
| Incubation period | *** | *** | ** | *** | ** | * | *** | *** | *** |
| Soil amendment × Incubation period | *** | *** | *** | *** | *** | *** | *** | *** | *** |

*Note*. * Significant at $P \leq 0.05$; ** Significant at $P \leq 0.01$; *** Significant at $P \leq 0.001$; EC = Electrical conductivity; CEC = Cation exchange capacity

## pH

As shown in Figure 2, all soil treatments showed a gradual reduction in soil pH from month 1 to month 3, except for the 1% composted *Azolla* treatment, which showed increased pH. At the end of the IP, the control resulted in significantly lower soil pH than the other soil treatments, except for the 3% fresh *Azolla* and 3% composted *Azolla* treatments.

## Electrical Conductivity

Figure 3 shows a continuous increase in soil EC during the IP for the 3% composted *Azolla*, which registered the highest soil EC compared to the control and other treatments at the end of the IP. The soil treated with 3, 6, and 9% fresh *Azolla* showed no significant differences throughout the IP.

## Total Carbon

Figure 4 shows no significant differences between the fresh *Azolla* treatments for soil total C at the end of the IP. However, the soil treated with 3% composted *Azolla* showed the highest total C throughout the IP. At the end of the IP, 3% composted *Azolla* resulted in the highest soil total C, followed

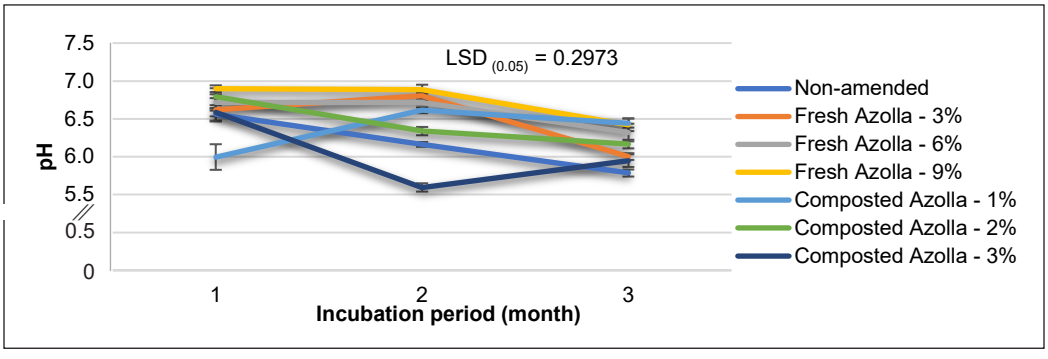



*Figure 2*. Interaction effects of soil amendments on soil pH during the incubation period
*Note*. Error bars represent the standard errors of the means

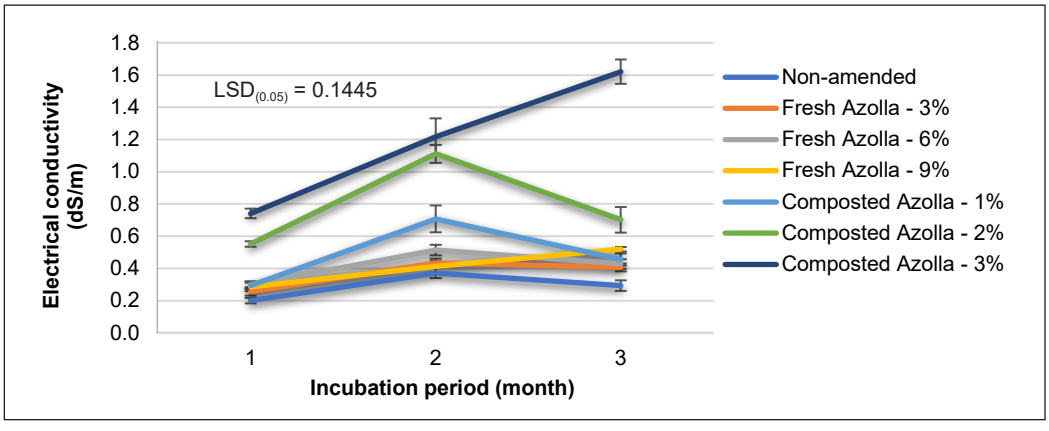



*Figure 3*. Interaction effects of soil amendments on soil electrical conductivity during the incubation period
*Note*. Error bars represent the standard errors of the means

by the control and 2% composted *Azolla* treatments.

## Total Nitrogen

Figure 5 shows that although there was a reduction in the total N of soil for all soil treatments at the end of the IP, the values were still above the initial total N values. The soil total N for the control and all soil treatments was reported to increase from the first month to the second month but decreased significantly in the third month of the IP. The 9% fresh *Azolla* treatment showed the highest total N compared to the other soil treatments in the second month of the IP. At the end of the IP, the soil treated with 3% composted *Azolla* showed significantly higher total N than the control and other soil treatments.

## Available Phosphorus

Figure 6 shows no significant differences between treatments for soil available P, except for the soil treated with 3% fresh

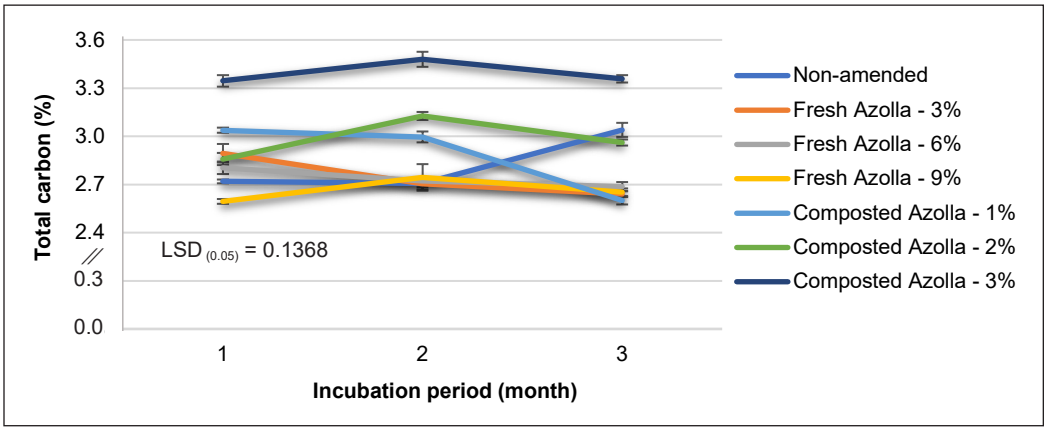



*Figure 4*. Interaction effects of soil amendments on soil total carbon during the incubation period
*Note*. Error bars represent the standard errors of the means

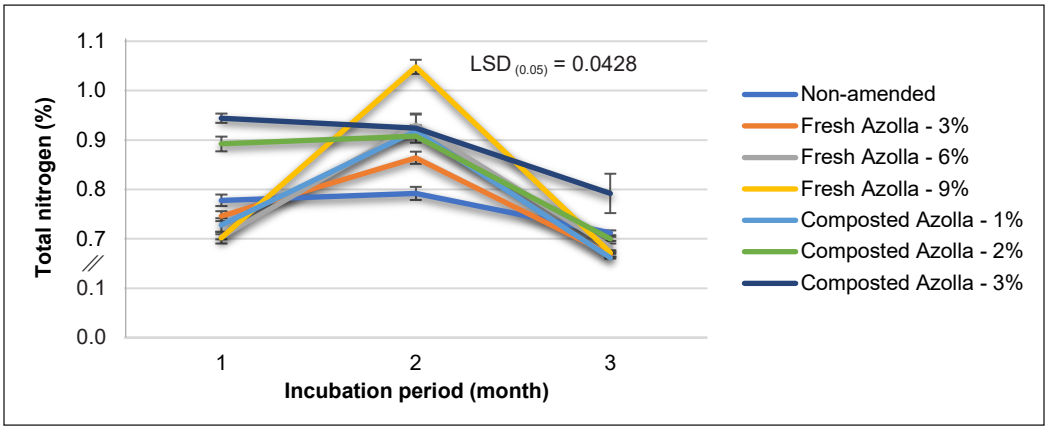



*Figure 5*. Interaction effects of soil amendments on soil total nitrogen during the incubation period
*Note*. Error bars represent the standard errors of the means

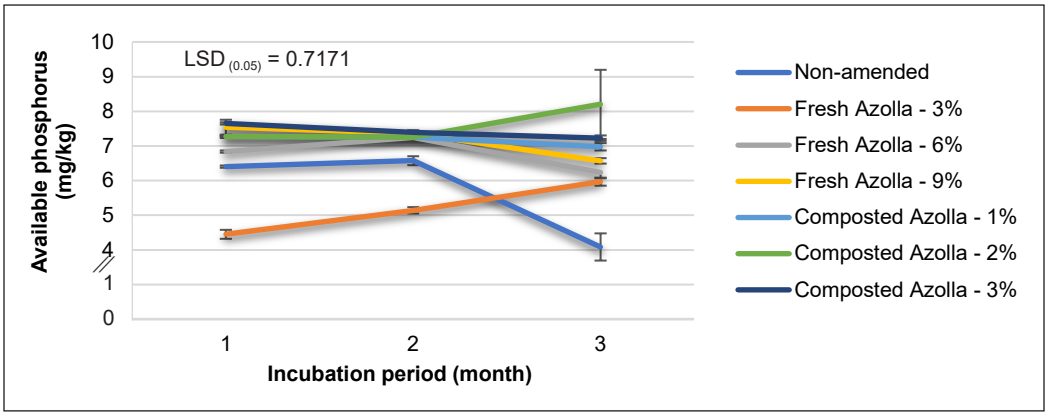



*Figure 6*. Interaction effects of soil amendments on soil available phosphorus during the incubation period
*Note*. Error bars represent the standard errors of the means

*Azolla* in the first and second months of the IP. At the end of the IP, the soil treated with 2% composted *Azolla* showed the highest available P compared to the control and other soil treatments, with an increasing trend observed throughout the IP.

### Exchangeable Potassium

As shown in Figure 7, during the first two months of the IP, the 3 and 6% fresh *Azolla* treatments showed no significant difference in exchangeable K as compared to the control, while the 1, 2, and 3% composted *Azolla* treatments showed the opposite. At the end of the IP, the 3% composted *Azolla* treatment resulted in significantly higher soil exchangeable K than the control, followed by the 2% composted *Azolla* treatment.

### Exchangeable Calcium

As seen in Figure 8, the control and all the *Azolla* treatments, except for the 3% fresh *Azolla* and 1% composted *Azolla* treatments, reduced exchangeable Ca throughout the IP. At the end of the IP, the 3% fresh *Azolla* treatment exhibited significantly higher exchangeable Ca than the non-amended soil, with an increasing trend throughout the IP, followed by the 9% fresh *Azolla* and 3% composted *Azolla* treatments.

### Exchangeable Magnesium

Figure 9 shows that exchangeable Mg for the fresh *Azolla* and 1% composted *Azolla* treatments was significantly reduced throughout the IP. In the third month of the IP, the non-amended soil had the highest exchangeable Mg compared to the other soil treatments. Further, the 2% and 3% composted *Azolla* treatments resulted in higher exchangeable Mg than the fresh *Azolla* treatments by the end of the IP.

### Cation Exchange Capacity

Figure 10 shows that in all soil treatments, except for the control, 6% fresh *Azolla* and 2% composted *Azolla*, the CEC increased from month 1 to month 3. At the end of the IP, however, the soil treated with 3% composted *Azolla* resulted in significantly

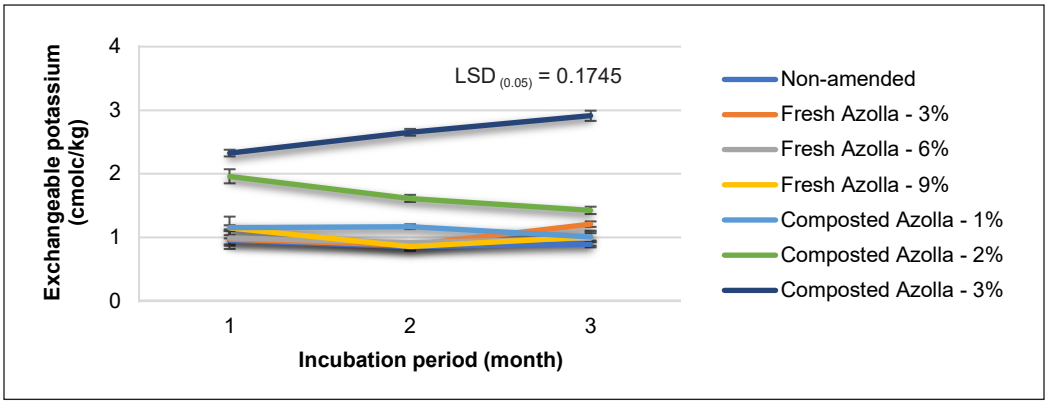



*Figure 7*. Interaction effects of soil amendments on soil exchangeable potassium during the incubation period
*Note*. Error bars represent the standard errors of the means

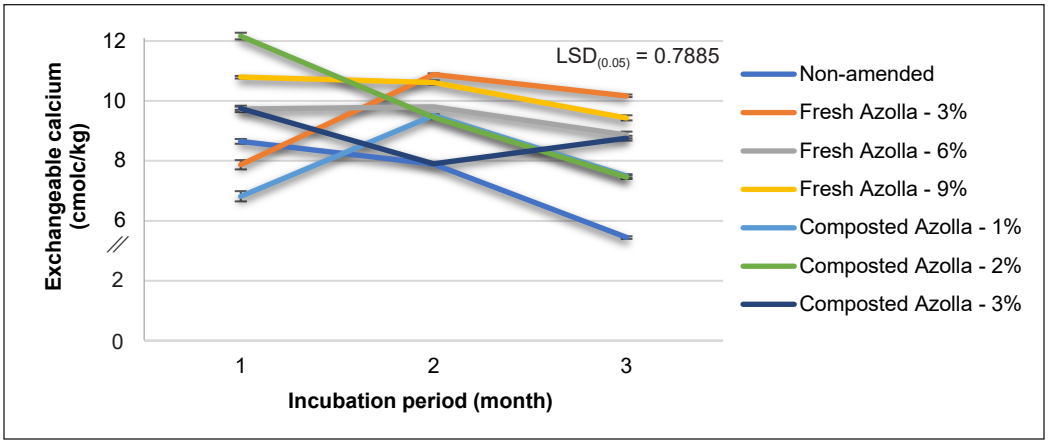



*Figure 8*. Interaction effects of soil amendments on soil exchangeable calcium during the incubation period
*Note*. Error bars represent the standard errors of the means

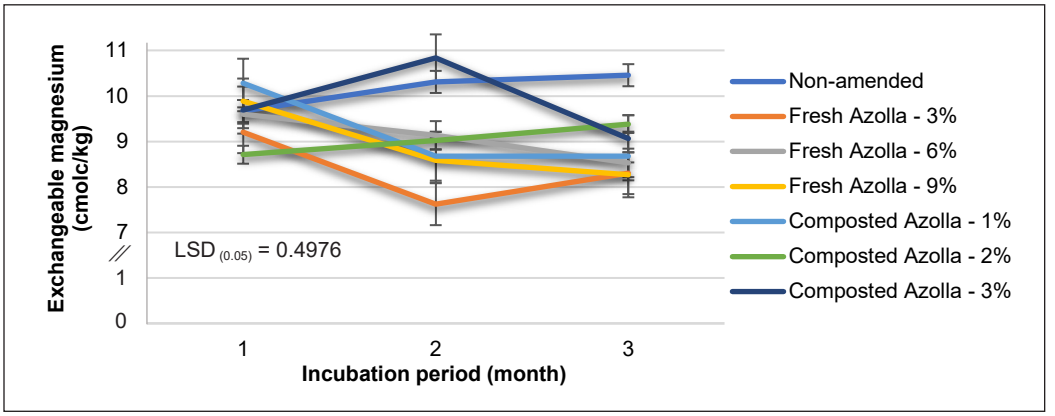



*Figure 9*. Interaction effects of soil amendments on soil exchangeable magnesium during the incubation period
*Note*. Error bars represent the standard errors of the means

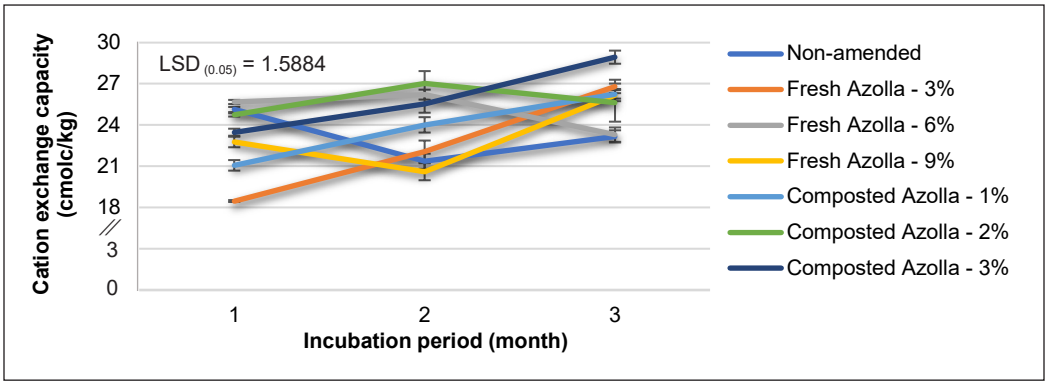



*Figure 10*. Interaction effects of soil amendments on soil cation exchange capacity during the incubation period
*Note*. Error bars represent the standard errors of the means

higher CEC, with a range of 7.36 to 19.46%, compared to the control and other soil treatments.

## DISCUSSION

Commonly, soil amendments promote plant growth and development in farming by supplying organic and inorganic nutrients to the soil and enhancing soil organic matter and water-holding capacity (Clements & Bihn, 2019). High organic matter content improves soil chemical properties, suppressing the solubility of aluminum (Al) and iron (Fe) in the soil through the process of complexation, pH buffering, precipitation, and competitive sorption (B. Sarkar et al., 2018; Ifansyah, 2013; Sung et al., 2017). Further, soil's physical and chemical characteristics are also influenced by clay minerals, the most reactive particles in soil. Clay minerals have abundant specific surface area and a net negative surface charge, allowing them to bond with and chemically stabilize organic matter (B. Sarkar et al., 2018). Generally, C components from organic matter adsorb onto clay minerals through mechanisms such as electrostatic attraction, hydrophobic attraction, ligand exchange, and π-bonding, protecting it from microbial decomposition (Baldock & Skjemstad, 2000; M. Singh et al., 2018). Consequently, applying compost to soils with high clay content will likely enhance C stabilization (Bolan et al., 2012). It explains the improvements in the clay soil's properties in this study.

Changes in soil pH are influenced by the release of basic cations such as Ca, Mg, K, and Na from weathered minerals, which leaves hydrogen ($H^+$) and Al ions as the dominant exchangeable cations; humic residues from the humification of soil organic matter, that results in large numbers of carboxyl and phenolic groups, which break down to release $H^+$ ions, nitrification of ammonium to nitrate resulting in the presence of $H^+$ ions, and the elimination of N in plant and animal products (Adeleke et al., 2017; Hong et al., 2019; White, 2005). Further, soil nutrients' solubility and availability greatly influence the soil's EC. The increase in soil EC throughout the

IP with the composted *Azolla* treatments was due to higher nutrient release from the composted organic matter than the fresh *Azolla* treatments. It resulted in more salts and ions in the soil and liquid phase, influencing soil EC over time (do Carmo et al., 2016). Thus, the higher the rates of organic matter applied, the higher the number of salts and ions released into the soil. As Iacomino et al. (2022) reported, soil EC can increase over time through compost applications alone.

Soil C and N are negatively associated with soil pH, indicating that a low pH promotes organic matter accumulation (Zhou et al., 2019). It explains the increase of total C and N and the associated decrease in pH of soil treated with *Azolla* biomass over the first two months of the IP. The soil total C and N for the 3% composted *Azolla* treatment was the highest at the end of the IP. These results are in line with Bharali et al. (2021), Benny et al. (2020), Novair et al. (2020), and Setiawati, Suryatmana, et al. (2018), where the incorporation of *Azolla* biomass, fresh or composted, increased soil total C and N compared to when there was no *Azolla* biomass applied. The *Azolla* biomass's high C and N content increases soil total C and N upon decomposition (Benny et al., 2020). Commonly, after four weeks of *Azolla* incorporation into the soil, 50% of the *Azolla* decomposes, releasing organic matter and nutrients into the soil (Ventura et al., 1992). Furthermore, soil C is positively correlated to soil N (Zhou et al., 2019). It explains the parallel increase of total C and N values in the second month of the soil IP. During the mineralization of organic matter, there is a constant and slow release of the fixed N stored in the *Azolla* leaves into the soil by the associated cyanobacteria (Seleiman et al., 2022).

On the other hand, the decrease in soil total N for all treatments at the end of the incubation period could be attributed to N losses through ammonia volatilization, denitrification or absorption of N by the moss plants that grew on the soil (Laruna et al., 2020).

As illustrated in Figure 6, at the end of the IP, the 2% composted *Azolla* treatment resulted in the highest amount of soil available P compared to the other soil treatments. Similar results were obtained by Sanjay-Swami and Singh (2019b) and Setiawati, Damayani, et al. (2018), who reported that *Azolla* biomass significantly increased soil P. The lower soil available P resulting from treatment with fresh *Azolla* may be related to the lower proliferation of microorganisms during the early stages of degradation and the potential immobilization of P by these microorganisms (Muktamar et al., 2020). Additionally, the *Azolla* biomass raised the value of P desorbed due to a primary reaction between the *Azolla* biomass and the soil, known as the acid neutralization reaction. This reaction lowers P fixation in acid soils and also encourages optimal nutrient utilization through timely nutrient delivery for maximum crop output (Johan et al., 2022).

Some soil chemical properties, such as the level of nutrients (N, P, K, Ca, Mg), show changes within a short period (<3

years) with all organic matter applications (Bhogal et al., 2018). Throughout the IP, the exchangeable K and Ca values of soil treated with 2 and 3% composted *Azolla* were higher than those for the fresh *Azolla* treatments. Further, soil treated with 3 and 9% fresh *Azolla* had higher exchangeable Ca compared to the other treatments at the end of the IP. Similar results were obtained by Rani et al. (2020), where *Azolla* biomass resulted in increased exchangeable K of about 50.91% from day 0 to day 35 of incubation and 3.31 and 4.05% from day 35 to 70 and day 70 to 105 of incubation, respectively. Moreover, Emam et al. (2022) stated that *Azolla* biomass organic amendment also increased the soil Ca and Mg levels.

*Azolla* biomass can supply nutrients as it has high N content and other minerals such as Ca, P, K, and Mg (Setiawati, Damayani, et al., 2018). It could be due to the decomposition of the fern, along with the effects on pH caused by the incorporation of *Azolla* biomass, which improved the solubility of these elements (Bhuvaneshwari & Singh, 2015). A further accelerated decrease in pH caused a reduction in the availability of exchangeable Ca and Mg. Throughout the IP, the soil pH range was 5.6 to 6.9, whereby the most abundant amount of Ca and Mg is found at pH <7.2. Additionally, the variations in soil exchangeable Mg and Ca concentrations were due to the soil exchangeable Al content (Miyazawa et al., 2001). The improved soil physical properties enhanced the mineralization of organic matter by the microorganisms in the soil pool, which possibly increased the exchangeable bases in the soil (Sanjay-Swami & Singh, 2019a, 2019b).

The soil treated with 3% composted *Azolla* showed the highest CEC compared to the other treatments at the end of the IP. This result is comparable with that obtained by Rani et al. (2020) and Sanjay-Swami and Singh (2019b). Changes in soil CEC are closely related to fluctuations in soil C content and the formation of negative charges in the soil organic matter and humified chemicals present in organic matter. Furthermore, clay soil is good at storing nutrients due to its high CEC (Pal & Marschner, 2016).

## CONCLUSION

Organic matter is crucial for restoring soil fertility and allaying concerns about soil damage from excessive chemical fertilizer use. The application of composted *Azolla*, especially 3% composted *Azolla*, improved the soil's chemical properties, which was most noticeable with soil EC, total C and N, exchangeable K, and CEC, compared to that of the non-amended soil and other *Azolla* amendments. Further, despite increasing application rates, fresh *Azolla* showed no substantial improvement in soil chemical properties, except for soil exchangeable Ca at the end of the IP. Based on the results from this experiment, composted *Azolla* helped the soil recover appropriate nutrient levels with only a single application over a 3-month incubation period. In future studies, it is recommended to consider other relevant soil analyses, such as soil bulk

density, to assess the effects of the applied *Azolla* amendments on the compactness of clay soils. Understanding how soil quality improvements affect crop growth is also essential in enhancing the reliability of the findings.

## ACKNOWLEDGEMENTS

The authors are grateful for all the facilities and research funds provided by Universiti Malaysia Sabah through grants GUG0518-2/2020 and SDN0069 to conduct this research.

## REFERENCES

Adeleke, R., Nwangburuka, C., & Oboirien, B. (2017). Origins, roles and fate of organic acids in soils: A review. *South African Journal of Botany*, *108*, 393–406. https://doi.org/10.1016/j.sajb.2016.09.002

AOAC International. (2002). *Determination of lead, cadmium, copper, iron, and zinc in foods: Atomic absorption spectrophotometry after dry ashing*. AOAC International.

Baldock, J. A., & Skjemstad, J. O. (2000). Role of the soil matrix and minerals in protecting natural organic materials against biological attack. *Organic Geochemistry*, *31*(7–8), 697–710. https://doi.org/10.1016/S0146-6380(00)00049-8

Barus, W. A., Khair, H., & Irawan, M. F. (2018). Growth response and production of broccoli (*Brassica oleracea*) with application of *Azolla* composting at several plant spacing. *Indonesian Journal of Agricultural Research*, *1*(2), 179–186. https://doi.org/10.32734/injar.v1i2.177

Benny, H., Sabrina, T. R. A., & Ali, J. (2020). Biochar and *Azolla* application on fertility of lead contaminated soil. *Russian Journal of Agricultural and Socio-Economic Sciences*, *9*(105), 134–142. https://doi.org/10.18551/rjoas.2020-09.15

Bernai, M. P., Paredes, C., Sánchez-Monedero, M. A., & Cegarra, J. (1998). Maturity and stability parameters of composts prepared with a wide range of organic wastes. *Bioresource Technology*, *63*(1), 91–99. https://doi.org/10.1016/S0960-8524(97)00084-9

Bharali, A., Baruah, K. K., Bhattacharya, S. S., & Kim, K. H. (2021). The use of *Azolla caroliniana* compost as organic input to irrigated and rainfed rice ecosystems: Comparison of its effects in relation to $CH_4$ emission pattern, soil carbon storage, and grain C interactions. *Journal of Cleaner Production*, *313*, 127931. https://doi.org/10.1016/j.jclepro.2021.127931

Bhogal, A., Nicholson, F. A., Rollett, A., Taylor, M., Litterick, A., Whittingham, M. J., & Williams, J. R. (2018). Improvements in the quality of agricultural soils following organic material additions depend on both the quantity and quality of the materials applied. *Frontiers in Sustainable Food Systems*, *2*, 9. https://doi.org/10.3389/fsufs.2018.00009

Bhuvaneshwari, K., & Singh, P. K. (2015). Response of nitrogen-fixing water fern *Azolla* biofertilization to rice crop. *3 Biotech*, *5*, 523–529. https://doi.org/10.1007/s13205-014-0251-8

Bolan, N. S., Kunhikrishnan, A., Choppala, G. K., Thangarajan, R., & Chung, J. W. (2012). Stabilization of carbon in composts and biochars in relation to carbon sequestration and soil fertility. *Science of the Total Environment*, *424*, 264–270. https://doi.org/10.1016/j.scitotenv.2012.02.061

Bray, R. H., & Kurtz, L. T. (1945). Determination of total, organic and available forms of phosphorus in soils. *Soil Science*, *59*(1), 39–46. https://doi.org/10.1097/00010694-194501000-00006

Bronick, C. J., & Lal, R. (2005). Soil structure and management: A review. *Geoderma*,

*124*(1–2), 3–22. https://doi.org/10.1016/j.geoderma.2004.03.005

Clements, D. P., & Bihn, E. A. (2019). The impact of food safety training on the adoption of good agricultural practices on farms. In D. Biswas & S. A. Micallef (Eds.), *Safety and practice for organic food* (pp. 321–344). Academic Press. https://doi.org/10.1016/B978-0-12-812060-6.00016-7

Djojosuwito, S. (2000). Azolla*: Pertanian organik dan multiguna* [*Azolla:* Organic and multipurpose agriculture]. Penerbit Kanisius.

do Carmo, D. L., de Lima, L. B., & Silva, C. A. (2016). Soil fertility and electrical conductivity affected by organic waste rates and nutrient inputs. *Revista Brasileira de Ciência Do Solo*, *40*, e0150152. https://doi.org/10.1590/18069657rbcs20150152

El-mrini, S., Aboutayeb, R., & Zouhri, A. (2022). Effect of initial C/N ratio and turning frequency on quality of final compost of turkey manure and Olive pomace. *Journal of Engineering and Applied Science*, *69*, 37. https://doi.org/10.1186/s44147-022-00092-6

Emam, M. S. A., Hawash, A. M. H., & Abul-Soud, M. A. (2022). Effect of some organic amendments on lettuce production under urban conditions. *Middle East Journal of Agriculture Research*, *11*(1), 134–145. https://doi.org/10.36632/mejar/2022.11.1.12

Ge, N., Wei, X., Wang, X., Liu, X., Shao, M., Jia, X., Li, X., & Zhang, Q. (2019). Soil texture determines the distribution of aggregate-associated carbon, nitrogen and phosphorous under two contrasting land use types in the Loess plateau. *CATENA*, *172*, 148–157. https://doi.org/10.1016/j.catena.2018.08.021

Hong, S., Gan, P., & Chen, A. (2019). Environmental controls on soil pH in planted forest and its response to nitrogen deposition. *Environmental Research*, *172*, 159–165. https://doi.org/10.1016/j.envres.2019.02.020

Iacomino, G., Sarker, T. C., Ippolito, F., Bonanomi, G., Vinale, F., Staropoli, A., & Idbella, M. (2022). Biochar and compost application either alone or in combination affects vegetable yield in a volcanic mediterranean soil. *Agronomy*, *12*(9), 1996. https://doi.org/10.3390/agronomy12091996

Ifansyah, H. (2013). Soil pH and solubility of aluminum, iron, and phosphorus in ultisols: The roles of humic acid. *Journal of Tropical Soil*, *18*(3), 203–208.

Johan, P. D., Ahmed, O. H., Hasbullah, N. A., Omar, L., Paramisparam, P., Hamidi, N. H., Jalloh, M. B., & Musah, A. A. (2022). Phosphorus sorption following the application of charcoal and sago (*Metroxylon sagu*) bark ash to acid soils. *Agronomy*, *12*(12), 3020. https://doi.org/10.3390/agronomy12123020

Jumadi, O., Hiola, S. F., Hala, Y., Norton, J., & Inubushi, K. (2014). Influence of *Azolla* (*Azolla microphylla* Kaulf.) compost on biogenic gas production, inorganic nitrogen and growth of upland kangkong (*Ipomoea aquatica* Forsk.) in a silt loam soil. *Soil Science and Plant Nutrition*, *60*(5), 722–730. https://doi.org/10.1080/00380768.2014.942879

Kalamdhad, A. S., & Kazmi, A. A. (2009). Rotary drum composting of different organic waste mixtures. *Waste Management and Research: The Journal for a Sustainable Circular Economy*, *27*(2), 129–137. https://doi.org/10.1177/0734242X08091865

Karam, D. S., Nagabovanalli, P., Rajoo, K. S., Ishak, C. F., Abdu, A., Rosli, Z., Muharam, F. M., & Zulperi, D. (2021). An overview on the preparation of rice husk biochar, factors affecting its properties, and its agriculture application. *Journal of the Saudi Society of Agricultural Sciences*, *21*(3), 149-159. https://doi.org/10.1016/j.jssas.2021.07.005

Khalib, S. N. B., Zakarya, I. A., & Izhar, T. N. T. (2020). The effect of low initial C:N ratio during composting of rice straw ash with food

waste in evaluating the compost quality. In *IOP Conference Series: Earth and Environmental Science* (Vol. 476, No. 1, p. 012144). IOP Publishing. https://doi.org/10.1088/1755-1315/476/1/012144

Laruna, M. A., Azman, E. A., & Ismail, R. (2020). Effect of rhinoceros beetle (*Oryctes rhinoceros*) larvae compost and vermicompost on selected soil chemical properties. *World Research Journal of Agricultural Sciences*, *7*(2), 201–208.

Lestari, S. U., Mutryarny, E., & Susi, N. (2019). *Azolla mycrophylla* fertilizer for sustainable agriculture: Compost and liquid fertilizer applications. *International Journal of Scientific and Technology Research*, *8*(7), 542–547.

Marzouk, S. H., Tindwa, H. J., Amuri, N. A., & Semoka, J. M. (2023). An overview of underutilized benefits derived from *Azolla* as a promising biofertilizer in lowland rice production. *Heliyon*, *9*(1), e13040. https://doi.org/10.1016/j.heliyon.2023.e13040

Miyazawa, M., Pavan, M. A., Ziglio, C. O., & Franchini, J. C. (2001). Reduction of exchangeable calcium and magnesium in soil with increasing pH. *Brazilian Archives of Biology and Technology*, *44*(2), 149–153. https://doi.org/10.1590/S1516-89132001000200007

Muktamar, Z., Lifia., & Adiprasetyo, T. (2020). Phosphorus availability as affected by the application of organic amendments in Ultisols. *SAINS TANAH - Journal of Soil Science and Agroclimatology*, *17*(1), 16–22. https://doi.org/10.20961/stjssa.v17i1.41284

Novair, S. B., Hosseini, H. M., Etesami, H., & Razavipour, T. (2020). Rice straw and composted *Azolla* alter carbon and nitrogen mineralization and microbial activity of a paddy soil under drying–rewetting cycles. *Applied Soil Ecology*, *154*, 103638. https://doi.org/10.1016/j.apsoil.2020.103638

Oades, J. M. (1988). The retention of organic matter in soils. *Biogeochemistry*, *5*, 35–70. https://doi.org/10.1007/BF02180317

Pal, S., & Marschner, P. (2016). Influence of clay concentration, residue C/N and particle size on microbial activity and nutrient availability in clay-amended sandy soil. *Journal of Soil Science and Plant Nutrition*, *16*(2), 350–361. https://doi.org/10.4067/S0718-95162016005000033

Pereira, A. L. (2018). The unique symbiotic system between a fern and a *Cyanobacterium*, *Azolla-Anabaena azollae*: Their potential as biofertilizer, feed, and remediation. In E. C. Rigobelo (Ed.), *Symbiosis*. IntechOpen. https://doi.org/10.5772/intechopen.70466

Pinero-Rodríguez, M. J., Fernández-Zamudio, R., Arribas, R., Gomez-Mestre, I., & Díaz-Paniagua, C. (2021). The invasive aquatic fern *Azolla filiculoides* negatively impacts water quality, aquatic vegetation and amphibian larvae in Mediterranean environments. *Biological Invasions*, *23*, 755–769. https://doi.org/10.1007/s10530-020-02402-6

Rani, M., Jha, A. K., Bihari, B., Kumar, A., & Kumar, A. (2020). An incubation experiment to study potassium fractions using *Azolla*, vermicompost and muriate of potash as potassium sources in inceptisol of Bihar. *International Journal of Current Microbiology and Applied Sciences*, *9*(5), 2573–2582. https://doi.org/10.20546/ijcmas.2020.905.294

Sanjay-Swami, & Singh, S. (2019a). Effect of nitrogen application through urea and *Azolla* on yield, nutrient uptake of rice and soil acidity indices in acidic soil of Meghalaya. *Journal of Environmental Biology*, *41*, 139–146. https://doi.org/10.22438/jeb/41/1/MRN-1133

Sanjay-Swami, & Singh, S. (2019b). Harnessing production potential of acidic soils: Impacts of *Azolla* (*Azolla pinnata*) bio-fertilizer and urea on rice (*Oryza sativa* L.) performance, temporal soil

P availability and acidity indices. *South Asian Research Journal of Agriculture and Fisheries*, *1*(1), 1–7. https://doi.org/10.36346/sarjaf.2019.v01i01.001

Sarkar, B., Singh, M., Mandal, S., Churchman, G. J., & Bolan, N. S. (2018). Clay minerals — Organic matter interactions in relation to carbon stabilization in soils. In C. Garcia, P. Nannipieri, & T. Hernandez (Eds.), *The future of soil carbon: Its conservation and formation* (pp. 71–86). Academic Press. https://doi.org/10.1016/B978-0-12-811687-6.00003-1

Sarkar, D., & Haldar, A. (2010). *Physical and chemical methods in soil analysis: Fundamental concepts of analytical chemistry and instrumental technique*. New Age International Pvt. Ltd.

Seleiman, M. F., Elshayb, O. M., Nada, A. M., Elleithy, S. A., Baz, L., Alhammad, B. A., & Mahdi, A. H. A. (2022). *Azolla* compost as an approach for enhancing growth, productivity and nutrient uptake of *Oryza sativa* L. *Agronomy*, *12*(2), 416. https://doi.org/10.3390/agronomy12020416

Setiawati, M. R., Damayani, M., Herdiyantoro, D., Suryatmana, P., Anggraini, D., & Khumairah, F. H. (2018). The application dosage of *Azolla pinnata* in fresh and powder form as organic fertilizer on soil chemical properties, growth and yield of rice plant. In *AIP Conference Proceedings* (Vol. 1927, No. 1, p. 030017). AIP Publishing. https://doi.org/10.1063/1.5021210

Setiawati, M. R., Suryatmana, P., Budiasih., Sondari, N., Nurlina, L., Kurnani, B. A., & Harlia, E. (2018). Utilization *Azolla pinnata* as substitution of manure to improve organic rice yield and paddy soil health. In *IOP Conference Series: Earth and Environmental Science* (Vol. 215, No. 1, p. 012006). IOP Publishing. https://doi.org/10.1088/1755-1315/215/1/012006

Shafiee, M. R., Aziz, N. A. A., Ahmad, B., Yusof, M. N. M., & Vun, C. T. (2021). *Kawalan rumpai pada tanaman padi organik* [Weed control in organic rice]. *Buletin Teknologi MARDI*, *25*, 127-135.

Singh, B. (2018). Are nitrogen fertilizers deleterious to soil health? *Agronomy*, *8*(4), 48. https://doi.org/10.3390/agronomy8040048

Singh, M., Sarkar, B., Sarkar, S., Churchman, J., Bolan, N., Mandal, S., Menon, M., Purakayastha, T. J., & Beerling, D. J. (2018). Stabilization of soil organic carbon as influenced by clay mineralogy. *Advances in Agronomy*, *148*, 33–84. https://doi.org/10.1016/bs.agron.2017.11.001

Singh, V. K., Malhi, G. S., Kaur, M., Singh, G., & Jatav, H. S. (2022). Use of organic soil amendments for improving soil ecosystem health and crop productivity. In H. S. Jatav & V. D. Rajput (Eds.), *Ecosystem services: Types, management and benefits* (pp. 259–277). Nova Science Publishers. https://doi.org/10.52305/PFZA6988

Soil Survey Staff. (2014). *Kellogg soil survey laboratory methods manual: Soil survey investigations report no. 42, version 5.0*. U.S. Department of Agriculture, Natural Resources Conservation Service.

Sung, C. T. B., Ishak, C. F., Abdullah, R., Othman, R., Panhwar, Q. A., & Aziz, M. M. A. (2017). Soil properties (physical, chemical, biological, mechanical). In M. A. Ashraf, R. Othman, & C. F. Ishak (Eds.), *Soils of Malaysia* (1st ed., pp. 103-154). CRC Press. https://doi.org/10.1201/b21934

Thapa, P., & Poudel, K. (2021). *Azolla*: Potential biofertilizer for increasing rice productivity, and government policy for implementation. *Journal of Wastes and Biomass Management*, *3*(2), 62–68. https://doi.org/10.26480/jwbm.02.2021.62.68

Trupiano, D., Cocozza, C., Baronti, S., Amendola, C., Vaccari, F. P., Lustrato, G., Di Lonardo, S., Fantasma, F., Tognetti, R., & Scippa, G. S. (2017). The effects of biochar and its combination with compost on lettuce (*Lactuca*

*sativa* L.) growth, soil properties, and soil microbial activity and abundance. *International Journal of Agronomy*, *2017*, 3158207. https://doi.org/10.1155/2017/3158207

van Hove, C., & Lejeune, A. (1996). Does *Azolla* have any future in agriculture? In M. Rahman, A. K. Podder, C. van Hove, Z. N. T. Begum, T. Heulin, & A. Hartmann (Eds)., *Biological nitrogen fixation associated with rice production* (pp. 83–94). Springer. https://doi.org/10.1007/978-94-015-8670-2_10

Ventura, W., Watanabe, I., & Mascarifia, G. B. (1992). Mineralization of *Azolla* N and its availability to wetland rice. *Soil Science and Plant Nutrition*, *38*(3), 505–516. https://doi.org/10.1080/00380768.1992.10415082

White, R. E. (2005). *Principles and practice of soil science: The soil as a natural resource* (4th ed.). John Wiley & Sons.

Widiastuti, D. P., Davis, J. G., & Gafur, S. (2018). Azolla *fertilizer as an alternative n source for red spinach production on alluvial and peat soils in west Kalimantan, Indonesia*. https://www.researchgate.net/publication/328899361_Azolla_fertilizer_as_an_alternative_N_source_for_red_spinach_production_on_alluvial_and_peat_soils_in_West_Kalimantan_Indonesia

Yadav, R. K., Abraham, G., Singh, Y. V, & Singh, P. K. (2014). Advancements in the utilization of *Azolla-Anabaena* system in relation to sustainable agricultural practices. *Proceedings of the Indian National Science Academy*, *80*(2), 301–316. https://doi.org/10.16943/ptinsa/2014/v80i2/55108

Zhou, W., Han, G., Liu, M., & Li, X. (2019). Effects of soil pH and texture on soil carbon and nitrogen in soil profiles under different land uses in Mun River Basin, Northeast Thailand. *PeerJ*, *7*, e7880. https://doi.org/10.7717/peerj.7880

Pertanika J. Trop. Agric. Sci. 47 (4): 1309 - 1323 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# The Vulnerary Potential of Malaysian Traditional Vegetables as Antibacterial Agents of Fish Pathogens: A Preliminary Study

**Rashidah Abdul Razak[1,2]*, Mohd Firdaus Nawi[1,2], Nur Izzati Farhanah Mohd Nasir[1], Nor Farhana Ayuni Abidin[1] and Nur Ajierah Jamaludin[1]**

*[1]Department of Marine Science, Kulliyyah of Science, International Islamic University Malaysia, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia*
*[2]Institute of Oceanography and Maritime Studies, Kulliyyah of Science, International Islamic University Malaysia, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia*

## ABSTRACT

Controlling antibiotic use in aquaculture demands the development of more sustainable alternative treatments for bacterial diseases. Therefore, the present study aims to evaluate the *in vitro* antibacterial effects of ethanolic extracts derived from ten popular and commonly consumed Malaysian traditional vegetables against *Aeromonas hydrophila*, *Aeromonas jandaei*, *Aeromonas sobria*, and *Edwardsiella tarda*. Various parts of plants were assessed for their inhibitory activity using disc diffusion, minimum inhibitory concentration, and minimum bactericidal concentration (MBC) methods. The *Persicaria odorata* and *Garcinia atroviridis* extracts extracted using the maceration method showed a wide range of inhibitory effects, but others showed less activity. *Aeromonas hydrophila* was the most susceptible bacterial strain, with all plant extracts suppressing its growth, while *A. sobria* is the most resistant strain. The minimum inhibition concentration (MIC) value ranged from 0.39 to 100 mg/ml, and all tested bacteria's MBC/MIC ratio was demonstrated to be bactericidal (MBC/MIC ratio <4). The findings of this study reveal the potential of *P. odorata* and *G. atroviridis* extracts as natural antibacterial agents that could be a safer and more effective alternative treatment in controlling bacterial infections in freshwater fish.

*Keywords*: Antibacterial activity, fish pathogenic bacteria, minimum bactericidal concentration, minimum inhibition concentration, traditional vegetable

ARTICLE INFO

*Article history:*
Received: 02 February 2024
Accepted: 02 May 2024
Published: 19 November 2024

DOI: https://doi.org/10.47836/pjtas.47.4.15

*E-mail addresses*:
rashidahrazak@iium.edu.my (Rashidah Abdul Razak)
firdausn@iium.edu.my (Mohd Firdaus Nawi)
izzatihana18@gmail.com (Nur Izzati Farhanah Mohd Nasir)
farhanaayuni08@gmail.com (Nor Farhana Ayuni Abidin)
ajierah98@gmail.com (Nur Ajierah Jamaludin)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

## INTRODUCTION

The evolution and spread of antibiotic resistance in fish pathogens pose substantial difficulties to the aquaculture sector and public health. The indiscriminate use of antibiotics in aquaculture can result in the growth of antibiotic-resistant bacteria and an excessive buildup of antibiotic residues in aquaculture products, especially fish (Okon et al., 2022). The use of antibiotics in aquaculture is not strictly controlled in many developing countries. Even in cases where rules are in place, strict adherence to these regulations is often absent. Fish producers in many countries unintentionally administer antibiotics without accurately determining the underlying causes of fish infection (Rahman et al., 2022). These practices jeopardise antibiotic effectiveness in treating bacterial infections in animals and humans and raise worries about the possible transmission of antibiotic-resistant bacteria from aquaculture products to consumers (Pepi & Focardi, 2021).

As a result, different strategies for controlling fish infections in aquaculture are urgently needed. Natural compounds such as polyphenols, alkaloids, terpenoids, and saponins (Silva & Fernandes Júnior, 2010) obtained from herbs or plants, which have been utilised for centuries in traditional medicine for their medicinal value, are one potential option. Malaysian traditional vegetables, with their diverse phytochemical profiles and abundant diversity (Ghasemzadeh et al., 2018; Maran et al., 2022; Mohd Noor et al., 2020), have the potential to provide natural antibacterial properties against fish infections. Moreover, it has been proven that plant extracts are less expensive, more readily available, simple to administer, and safer than commercial antibiotics (Vaou et al., 2021). Most importantly, they are effective, safer and have fewer side effects on the treated aquatic animals (Guo et al., 2023; Zhang et al., 2022).

Plant extracts have been shown to have antibacterial activity against various bacterial strains that are important in aquaculture productivity. A recent study by Tkachenko et al. (2023) determined the antibacterial properties in the leaf extracts of *Ficus elastica* and its cultivars, which inhibited the growth of *A. sobria*, *A. hydrophila*, and *Aeromonas salmonicida* subsp. *salmonicida*. The leaf extracts of various *Ficus* spp. also demonstrated antimicrobial action against other fish bacterial pathogens, including *Serratia liquefaciens*, *Yersinia ruckeri*, *Pseudomonas fluorescens*, and *Shewanella putrefaciens* (Tkachenko et al., 2022). Essential oils derived from natural plants such as *Eucalyptus camaldulensis* (Bektaş & Özdal, 2022), *Gaultheria procumbens*, *Litsea cubeba* (Klūga et al., 2021), *Cinnamomum cassia* (Junior et al., 2022), *Origanum vulgare* subsp. *hirtum*, *Thymbra capitata*, and *Satureja thymbra* (Anastasiou et al., 2020) exhibited strong inhibitory activity against the growth of aquaculture bacterial pathogens.

Little research has been done on the antibacterial properties of popular and commonly consumed Malaysian traditional

vegetables against aquaculture pathogens *in vitro* and *in vivo*. Therefore, this study aimed to explore the *in vitro* antibacterial efficiency of ethanol extracts derived from ten Malaysian traditional vegetables against the common fish bacterial pathogens, *A. hydrophila*, *A. sobria*, *A. jandaei*, and *E. tarda* that affect freshwater fish aquaculture productions.

## MATERIALS AND METHODS

### Plant Materials and Extract Preparation

Fresh part of ten Malaysian traditional vegetables were sourced from Kuantan, Pahang, Malaysia, with details shown in Table 1 and Figure 1. The specimens were identified by a botanist of the Department of Plant Science, Kulliyyah of Science (KOS), International Islamic University Malaysia (IIUM), Dr. Rozilawati Shahari, and voucher specimens were deposited at the herbarium, KOS, IIUM. Each plant material was cleaned and dried at 40ºC for 2–3 days. The dry materials were crushed into powder and stored in dark, sealed bags. Each plant's powdered materials were weighed into a 500 ml conical flask, and 80% ethanol (HmbG Chemicals, Malaysia) was added with a ratio of 1:10 (w/v) before macerating for 48 hr. After centrifuging the mixtures 300 × *g* for 10 min, they were filtered using Whatman No. 1 filter paper. The collected filtrates were subjected to rotary evaporation (BUCHI R-100 equipped with vacuum pump V-100 and Interface I-100, Germany) at 40ºC, aiming to eliminate the solvent from the extract.

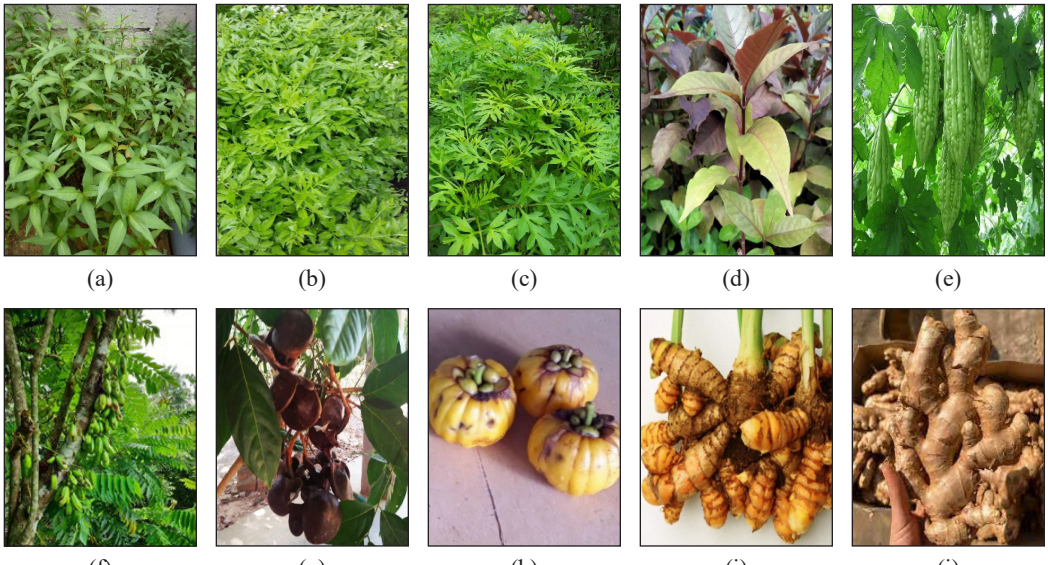



*Figure 1*. Plant parts used in this study: (a) leaf of *Persicaria odorata*; (b) leaf of *Oenanthe javanica*; (c) leaf of *Cosmos caudatus;* (d) leaf of *Gynura bicolour*; (e) leaf and fruit of *Momordica charantia*; (f) leaf and fruit of *Averrhoa bilimbi*; (g) leaf and legume of *Pithecellobium jiringa*; (g) the fruit of *Garcinia atroviridis*; (i) rhizome of *Curcuma longa*; and (j) rhizome of *Zingiber officinale*

Table 1
*Description of plant extracts used in this study*

| Scientific name | Common name | Botanical family | Part used |
|---|---|---|---|
| *Persicaria odorata* | Vietnamese coriander | Polygonaceae | Leaf |
| *Oenanthe javanica* | Chinese celery, Japanese parsley | Apiaceae | Leaf |
| *Cosmos caudatus* | King's salad | Asteraceae | Leaf |
| *Gynura bicolor* | Okinawan spinach | Asteraceae | Leaf |
| *Momordica charantia* | bitter melon | Cucurbitaceae | Leaf and fruit |
| *Averrhoa bilimbi* | Bilimbi | Oxalidaceae | Leaf and fruit |
| *Pithecellobium jiringa* | Jering | Fabaceae | Leaf and legume |
| *Garcinia atroviridis* | Asam Gelugur, asam keping | Clusiaceae | Fruit |
| *Zingiber officinale* | Ginger | Zingiberaceae | Rhizome |
| *Curcuma longa* | Turmeric | Zingiberaceae | Rhizome |

The leftover solvent was evaporated further in the oven at 40ºC, and the crude extracts were kept in dark containers at -20ºC before use (Eruygur et al., 2019).

## Test Organisms and Preparation of Inoculum

*Aeromonas hydrophila* K3T8, *A. sobria* K1P2, *A. jandaei* K2P2, and *E. tarda* K3P2 were isolated from kidneys of diseased Pangasius catfish (*Pangasius hypophthalmus*) and red hybrid tilapis (*Oreochromis* spp.) and identified using 16S rRNA and specific genes (Hussain et al., 2014; Sakai et al., 2009) polymerase chain reaction (PCR). The cultures were maintained in tryptic soy agar (TSA, Merck, Germany). A loopful of 24-hr-old cultures were inoculated in 9 ml sterile 0.9% saline water (Fisher Scientific, USA). Each inoculum was prepared with a cell concentration of $1.5 \times 10^8$ CFU/ml by comparing with 0.5 McFarland standard (ThermoFisher Scientific, USA) to obtain a standardised inoculum for the tests.

## Antibacterial Activity

### *Disc Diffusion Assay*

The disc diffusion method was used to assess the antibacterial activity of the extracts on fish pathogenic bacteria. The experiment complied with the Clinical and Laboratory Standards Institute (CLSI) recommendations (Clinical and Laboratory Standards Institute, 2018). Briefly, on the surface of sterile Mueller Hinton agar (MHA, Merck, Germany), 100 µl of fresh bacterial inoculum (approximately 1-2 $\times 10^6$ CFU/ml according to McFarland standards) was swabbed uniformly and left to dry. Then, sterilised filter paper discs (6 mm diameter, Whatman, Merck, Germany) were placed on the surface of the MHA after being impregnated with 20 µl of each extract (60 µg per disc) dissolved in 2% dimethyl sulfoxide (DMSO, Fisher Scientific, USA) and left to dry for 15 min. Negative control was disc embedded with 2% DMSO, whereas positive controls were tetracycline, chloramphenicol, erythromycin, and

oxytetracycline (50 µg per disc, Calbiochem, USA). The bacteria-inoculated plates were incubated inverted at 37ºC for 24 hr. After incubation, the diameters (mm) of the inhibition zones around the discs were measured and performed in triplicate.

### *The Determination of MIC*

A subset of antibacterial action extracts was evaluated for the MIC and MBC. The broth microdilution method determined the MIC of plant extracts against the bacterial strains according to the CLSI guidelines (CLSI, 2018). In 96-well microtiter plates, the extracts were serially diluted twofold in Mueller Hinton broth (MHB, Merck, Germany) to generate final concentrations ranging from 0.20 to 200 mg/ml. Subsequently, a comparable 50 µl bacterial suspension volume was introduced into each well with an estimated $10^6$ CFU/ml concentration. Following incubation at 37ºC for 16–18 hr, the bacterial growth was detected by assessing medium turbidity. The MIC refers to the lowest concentration of the extracts necessary to prevent bacterial growth without causing turbidity in the medium, as shown in the negative control (wells contain media only). The positive control wells contain bacterial suspension without any extracts. The experiments were conducted three times for each assay.

### *The Determination of the MBC*

The wells that exhibited bacterial inhibition in MIC, including the positive (wells contain bacterial suspension without any extracts) and negative (wells contain media only) controls, were subjected to plating on MHA using 10 µl aliquots of the contents. The plated samples were then incubated at 37ºC for 16–24 hr, as described by Junior et al. (2019). The MBC was determined as the concentration of the extracts at which no visible growth of bacteria was evidenced on MHA. The experiments were conducted in triplicate.

### Statistical Analysis

The data were presented as means ± standard errors (SE). The statistical analysis utilised a one-way analysis of variance (ANOVA) and a post hoc Duncan's test, all conducted using SPSS Statistical 26 software. A significance level of $p < 0.05$ was deemed significant for all statistical tests.

## RESULTS

### Zone of Inhibition

Table 2 shows the results of the antibacterial screening assays as determined by the disc diffusion method. The antibacterial potential of plant extracts varied significantly ($p$ = 0.00) depending on the type of plant and bacterial strain. The plant extracts displayed inhibitory zones with diameters ranging from 6.77 to 26.33 mm, with the most remarkable outcomes observed from *P. odorata* and *G. atroviridis*.

*Persicaria odorata* and *G. atroviridis* extracts exhibited a broad spectrum of inhibitory effects due to their significant antibacterial activity (inhibition zones of ≥ 15.00 mm) against the four tested bacterial strains, whereas the other extracts exhibited

Table 2
*Antibacterial activity of the plant extracts as measured by disc diffusion assay*

| | | The mean diameter of the inhibition zones (mm ± SE) | | | |
|---|---|---|---|---|---|
| | | ***Aeromonas hydrophila*** | ***Aeromonas sobria*** | ***Aeromonas jandaei*** | ***Edwardsiella tarda*** |
| Plant species | Part | | | | |
| *Persicaria odorata* | Leaves | $20.53 \pm 0.09^{f,C}$ | $26.33 \pm 0.33^{d,D}$ | $17.33 \pm 0.33^{,B}$ | $15.00 \pm 0.12^{c,A}$ |
| *Oenanthe javanica* | Leaves | $8.47 \pm 0.03^{bc,A}$ | NA | ND | $10.03 \pm 0.03^{b,B}$ |
| *Cosmos caudatus* | Leaves | $10.03 \pm 0.09^{d,B}$ | NA | ND | $9.47 \pm 0.03^{b,A}$ |
| *Gynura bicolor* | Leaves | $10.07 \pm 0.07^{d,A}$ | NA | ND | NA |
| *Momordica charantia* | Leaves | $7.77 \pm 0.14^{ab,B}$ | NA | $8.97 \pm 0.09^{b,C}$ | $6.77 \pm 0.15^{a,A}$ |
| *Momordica charantia* | Fruit | $7.37 \pm 0.03^{ab,B}$ | NA | $7.20 \pm 0.17^{a,AB}$ | $6.87 \pm 0.20^{a,A}$ |
| *Averrhoa bilimbi* | Leaves | $7.53 \pm 0.20^{ab,B}$ | NA | $7.10 \pm 0.09^{a,A}$ | NA |
| *Averrhoa bilimbi* | Fruit | $9.13 \pm 0.07^{cd,B}$ | $8.00 \pm 0.17^{a,A}$ | $7.53 \pm 0.20^{ab,A}$ | $9.27 \pm 0.26^{b,B}$ |
| *Pithecellobium jiringa* | Leaves | $7.00 \pm 0.00^{a,A}$ | NA | $7.10 \pm 0.23^{a,A}$ | $8.53 \pm 0.37^{b,B}$ |
| *Pithecellobium jiringa* | Fruit | $7.17 \pm 0.20^{ab,A}$ | $8.50 \pm 0.17^{a,B}$ | $7.43 \pm 0.24^{ab,A}$ | NA |
| *Garcinia atroviridis* | Fruit | $16.33 \pm 0.88^{e,AB}$ | $26.33 \pm 0.33^{d,C}$ | $17.33 \pm 1.33^{d,B}$ | $13.50 \pm 0.76^{e,A}$ |
| *Zingiber officinale* | Rhizome | NA | $12.00 \pm 0.58^{b,A}$ | NA | NA |
| *Curcuma longa* | Rhizome | NA | $13.67 \pm 0.88^{c,A}$ | NA | NA |
| Controls | | | | | |
| Erythromycin* | | $20.00 \pm 1.15^{f,A}$ | $25.67 \pm 0.88^{d,B}$ | $25.00 \pm 1.15^{e,B}$ | $32.00 \pm 1.73^{e,C}$ |
| Tetracycline* | | $28.03 \pm 0.20^{g,B}$ | $13.40 \pm 0.17^{c,A}$ | $13.10 \pm 0.06^{c,A}$ | $27.90 \pm 0.47^{d,B}$ |
| Chloramphenicol* | | $29.13 \pm 0.35^{g,AB}$ | $29.90 \pm 0.21^{e,B}$ | $32.00 \pm 0.58^{f,C}$ | $28.33 \pm 0.29^{d,A}$ |
| Oxytetracycline* | | $44.33 \pm 0.88^{h,C}$ | $31.67 \pm 0.67^{f,A}$ | $35.00 \pm 1.15^{g,B}$ | $33.33 \pm 0.88^{e,AB}$ |
| 2% DMSO** | | NA | NA | NA | NA |

*Note*. Different lowercase letters indicate differences between plant extracts within each pathogen, and different capital letters indicate differences between pathogens within each plant extract ($p < 0.05$). Inhibition zones > 15 mm were categorised as strong activity, 8 to 15 mm as moderate activity, and 1 to 8 mm as weak activity; SE = Standard error; NA = No growth inhibition; ND = Not tested; DMSO = Dimethyl sulfoxide; * = Positive control; ** = Negative control

limited antibacterial activity. However, the antibacterial potential of *Z. officinale* and *C. longa* extracts against *A. sobria*, *C. caudatus*, and *G. bicolor* extracts against *A. hydrophila* was observed since their inhibition zones were more than 10.00 mm.

*Aeromonas hydrophila* was this study's most susceptible bacterial strain, with all plant extracts inhibiting its growth. This pathogen is significantly suppressed by *P. odorata* and *G. atorividis* extracts, moderately inhibited by *O. javanica*, *C. caudatus*, *G. bicolor*, and *A. bilimbi* extracts, and poorly inhibited by the other five extracts. Meanwhile, *A. sobria* is the most resistant strain, with no inhibition zone observed in seven plant extracts and only faintly inhibited (8.00 ± 0.17 mm) by *A. bilimbi* extract.

The negative control (2% DMSO) had no inhibition zone. In contrast, the three positive controls (erythromycin, chloramphenicol, and oxytetracycline) had strong antibacterial action against all pathogens tested (inhibition zones ranging from 20.0 to 44.33 mm). Meanwhile, one reference antibiotic (tetracycline) showed significant antibacterial action against *A. hydrophila* (28.03 ± 0.20 mm) and *E. tarda* (27.90 ± 0.47 mm), but only moderate activity against *A. sobria* (13.40 ± 0.17 mm) and *A. jandaei* (13.10 ± 0.06 mm).

## MIC

The MIC values of the crude extracts that showed positive results in the antibacterial activity tests are presented in Tables 3 to 6, ranging from 0.39 to 100.00 mg/ml. *Persicaria odorata* extract demonstrated the lowest MIC against all tested bacteria (MIC 0.39 mg/ml), followed by *G. atroviridis* extract, which had MICs of 1.56 mg/ml against *A. sobria*, *A. jandaei*, and *E. tarda*, and MIC of 6.25 mg/ml against *A. hydrophila*.

A weak inhibition action was observed in fruit and leaf extracts of *M. charantia* (MIC of 50.00 and 100.00 mg/ml, respectively) and fruit and leaf extracts of *A. bilimbi* against *A. hydrophila* (MIC = 50.00 mg/ml), rhizome extract of *C. longa* and fruit extracts of *A. bilimbi* and *P. jiringa* against *A. sobria* (MIC = 50.00 mg/ml), leaf extract of *M. charantia* and fruit extract of *A. bilimbi* against *A. jandaei* and *E. tarda* (MIC = 50.00 mg/ml, respectively). However, no inhibitory action was identified in *P. jiringa* fruit and leaf extracts against *A. hydrophila* and *A. jandaei* and *M. charantia* fruit and leaf extracts against *A. jandaei* and *E. tarda*.

## Mode of Action and MBC

The MBC values ranged from identical to the MIC to substantially lower, as shown in Tables 3 to 6. Similar to the MIC result, *P. odorata* ethanolic extract showed significantly higher bactericidal activity against all tested bacteria (MBC value of 0.20 mg/ml). Based on the CLSI (2018), a drug is considered to exhibit bactericidal activity when the MBC/MIC ratio is $\leq 4$. In contrast, the drug is bacteriostatic when the corresponding MBC/MIC ratio $\geq 8$. According to the MBC/MIC ratio results, all extracts with positive MIC findings were bactericidal against all tested bacteria

Table 3
*Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values of plant extracts on* Aeromonas hydrophila

| Plant | Part | MIC (mg/ml) | MBC (mg/ml) | MBC/MIC |
|---|---|---|---|---|
| *Persicaria odorata* | Leaves | 0.39 | 0.39 | 1 |
| *Oenanthe javanica* | Leaves | 12.50 | 12.50 | 1 |
| *Cosmos caudatus* | Leaves | 1.56 | 1.56 | 1 |
| *Gynura bicolor* | Leaves | 0.78 | 0.78 | 1 |
| *Momordica charantia* | Leaves | 50.00 | 100.00 | 2 |
| *Momordica charantia* | Fruit | 100.00 | 100.00 | 1 |
| *Averrhoa bilimbi* | Leaves | 50.00 | 100.00 | 2 |
| *Averrhoa bilimbi* | Fruit | 50.00 | 50.00 | 1 |
| *Pithecellobium jiringa* | Leaves | NA | ND | ND |
| *Pithecellobium jiringa* | Fruit | NA | ND | ND |
| *Garcinia atroviridis* | Fruit | 6.25 | 6.25 | 1 |

*Note*. NA = No growth inhibition; ND = Not tested

Table 4
*Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values of plant extracts on* Aeromonas sobria

| Plant | Part | MIC (mg/ml) | MBC (mg/ml) | MBC/MIC |
|---|---|---|---|---|
| *Persicaria odorata* | Leaves | 0.39 | 0.39 | 1 |
| *Averrhoa bilimbi* | Fruit | 50.00 | 50.00 | 1 |
| *Pithecellobium jiringa* | Fruit | 50.00 | 50.00 | 1 |
| *Garcinia atroviridis* | Fruit | 1.56 | 1.56 | 1 |
| *Zingiber officinale* | Rhizome | 12.50 | 12.50 | 1 |
| *Curcuma longa* | Rhizome | 50.00 | 100.00 | 2 |

*Note*. NA = No growth inhibition; ND = Not tested

Table 5
*Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values of plant extracts on* Aeromonas jandaei

| Plant | Part | MIC (mg/ml) | MBC (mg/ml) | MBC/MIC |
|---|---|---|---|---|
| *Persicaria odorata* | Leaves | 0.39 | 0.39 | 1 |
| *Momordica charantia* | Leaves | 50.00 | 100.00 | 1 |
| *Momordica charantia* | Fruit | NA | ND | ND |
| *Averrhoa bilimbi* | Leaves | NA | ND | ND |
| *Averrhoa bilimbi* | Fruit | 50.00 | 100.00 | 2 |
| *Pithecellobium jiringa* | Leaves | NA | ND | ND |
| *Pithecellobium jiringa* | Fruit | NA | ND | ND |
| *Garcinia atroviridis* | Fruit | 1.56 | 1.56 | 1 |

*Note*. NA = No growth inhibition; ND = Not tested

Table 6
*Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values of plant extracts on* Edwardsiella tarda

| Plant | Part | MIC (mg/ml) | MBC (mg/ml) | MBC/MIC |
|---|---|---|---|---|
| *Persicaria odorata* | Leaves | 0.39 | 0.39 | 1 |
| *Oenanthe javanica* | Leaves | 1.56 | 1.56 | 1 |
| *Cosmos caudatus* | Leaves | 0.78 | 1.56 | 2 |
| *Momordica charantia* | Leaves | NA | ND | ND |
| *Momordica charantia* | Fruit | NA | ND | ND |
| *Averrhoa bilimbi* | Fruit | 50.00 | 50.00 | 1 |
| *Pithecellobium jiringa* | Leaves | 25.00 | 50.00 | 2 |
| *Garcinia atroviridis* | Fruit | 1.560 | 1.56 | 1 |

*Note*. NA = No growth inhibition; ND = Not tested

(MBC/MIC ratio < 4). Most extracts had a comparable bactericidal effect, as evidenced by the MBC being equivalent to the MIC (MBC = MIC). In contrast, six extracts demonstrated a bactericidal mechanism of action when administered at concentrations beyond the MIC (MBC > MIC). The results indicate that the MBC values for *A. hydrophila* were slightly higher (100.00 mg/ml; MIC 50.00 mg/ml) when using the leaf extracts from both *M. charantia* and *A. bilimbi* (Table 3). Similarly, the MBC values showed minimal variations for *A. sobria* (100.00 mg/ml; MIC 50.00 mg/ml) when treated with *C. longa* and *A. jandaei* (100.00 mg/ml; MIC 50.00 mg/ml) using the fruit extract of *A bilimbi* (Tables 4 and 5). As for *E. tarda*, the MBC values were slightly elevated in *C. caudatus* (1.56 mg/ml; MIC 0.78 mg/ml) and *P. jiringa* (50.00 mg/ml; MIC 25.00 mg/ml) (Table 6).

## DISCUSSION

The aquaculture sector has long relied on antibiotics as a strategy to control infectious bacterial diseases. However, in light of the rise of antibiotic resistance and the consequential environmental impacts associated with their utilisation, there is a need for alternative approaches. Plants and their derivatives have exhibited considerable potential as viable alternatives to antibiotics in the treatment of bacterial infections. Their application is gaining popularity because of its convenient preparation process, low cost, less adverse effects, and environmental impact (Bondad-Reantaso et al., 2023).

This study presents the findings on the antibacterial properties of some Malaysian traditional vegetables, highlighting their potential as a viable substitute for antibiotics in managing the proliferation of fish pathogens, including *A. hydrohila*, *A. sobria*, *A. jandaei*, and *E. tarda*. Among them, *P. odorata* and *G. atroviridis* extracts had the highest activity against the tested pathogens. *Aeromonas hydrophila* and *A. sobria* showed susceptibility (IZ ≥ 20 mm), whereas *A. jandaei* and *E. tarda* exhibited moderate susceptibility to *P.*

*odorata* extract in the present study at 300 mg/ml concentration. In a study conducted by Ridzuan et al. (2013), the effectiveness of *P. odorata* extract at different concentrations (ranging from 50 to 400 mg/ml) was compared against eight bacterial strains. The results showed that both American Type Culture Collection (ATCC) and wild strains of *Streptococcus aureus* and *Streptococcus epidermis* were susceptible to the extract. However, both strains of *Streptococcus pyogenes* showed intermediate susceptibility to *P. odorata* at the highest concentration of 400 mg/ml. These results indicate that plant extract concentration plays a crucial role in determining its effectiveness in combating specific bacteria. Based on phytochemical analysis by Nguyen et al. (2020), *P. odorata* leaves contained various phytochemical compounds such as alkaloids, tannins, anthraquinone, flavonoids, terpenoids, coumarins, saponin, and other reducing compounds, which indicated its potential as antibacterial agents. However, the concentration of these bioactive compounds in the extract varies according to multiple factors, including selection of solvents, extraction techniques, plant materials and extraction time (AL Ubeed et al., 2022).

*Persicaria odorata*, commonly used in cuisines and has various medical properties, has also been reported to contain pharmacological activities such as antioxidants, anti-inflammatory, antibacterial, and anticancer effects (Azmi et al., 2021). The disc diffusion and time-kill assays revealed that silver nanoparticles synthesised from *P. odorata* inhibited methicillin-resistant *Staphylococcus aureus*, *S. aureus*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa*. These findings support the notion that *P. odorata* has antibacterial characteristics (Aminullah et al., 2021). Similarly, Řebíčková et al. (2020) demonstrated the antimicrobial efficiency of *P. odorata* essential oils against various bacteria, including both Gram-negative and Gram-positive strains. Specifically, these essential oils exhibited effectiveness against *Escherichia coli*, *S. aureus*, *P. aeruginosa*, *Enterococcus faecalis*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Seratia marcescense*, and *Bacillus subtilis*. These findings are similar to and further support the results of this study. Our antibacterial study of *P. odorata* extract yielded remarkably similar results of 15.00 to 26.33 mm inhibition zones, showcasing the consistent effectiveness against four common fish bacterial pathogens.

Our investigation into the antibacterial potential of *G. atroviridis* extract revealed results similar to those of *P. odorata* extract. Organic acids and flavonoids have been identified as the primary components of *G. atroviridis* extracts (Shahid et al., 2022), and some of these were reported to have biological activities (Reang et al., 2021; Schobert & Biersack, 2019; Tan et al., 2016). A variety of studies undertaken by several research groups have proved *G. atroviridis*' antibacterial ability. A recent study by Thongkham et al. (2021) investigated the antimicrobial activity of the ethanolic extract derived from *G.*

*atroviridis* fruit. The study demonstrated the effectiveness of this extract against various microorganisms, including *E. coli* TISTR 073, *Streptococcus agalactiae* ATCC 27956, *B. subtilis* DMST 3763, *Streptococcus intermedius* DMST 5024, *S. epidermidis* DMST 12853, *S. aureus* DMST 4745, *Candida albicans* ATCC 10, 231, and *Malassez pachydermatis*. Later, Niyomdecha et al. (2022) also synthesised *G. atroviridis* fruit ethanolic extract and discovered that it exhibited inhibitory effects against *E. coli* and *S. enterica* with inhibition zones of 33.11 and 31.58 mm, respectively, at a dosage of 200 mg/ml. Tan et al. (2019) conducted a similar study and discovered that stem bark extracts of *G. atroviridis* have inhibitory activities against food-borne bacteria, *Proteus mirabilis*, *S. epidermidis*, *Bacillus cereus*, *B. subtilis*, methicillin-resistant *S. aureus*, *E. coli*, *Citrobacter freundii*, *P. aeruginosa*, *K. pneumonia*, *Shigella boydii*, and anti-yeast capabilities against *Candida utilis*.

The MBC results in this study corresponded to the low MIC value. *Persicaria odorata* extract exhibited potentially bactericidal activity against all pathogenic bacteria tested (*A. hydrophila*, *A. sobria*, *A. jandaei*, and *E. tarda*) with MBC of 0.20 mg/ml, whereas *G. atroviridis* extract exhibited MBC of 0.78 mg/ml, except for *A. hydrophila*, which was less sensitive and had MBC of 3.13 mg/ml. The MIC and MBC outcomes obtained from evaluating plant extracts with antimicrobial properties indicate that *P. odorata* and *G. atroviridis* can be utilised to control and prevent fish bacterial infections. These results are in accordance with those of Bacayo et al. (2018), Niyomdecha et al. (2022), Řebíčková et al. (2020), Tan et al. (2019). The wide range in MIC of *P. odorata* and *G. atroviridis* extracts seen in many studies could be attributed to differences in the extraction process, solvents, and bacterial strains utilised. Variations in MIC of different plant extracts may also result from differences in their chemical compositions and the volatile nature of those components (Mostafa et al., 2018).

As far as the authors are aware, there have been no earlier findings on *G. atroviridis* extract as an antibacterial agent against fish pathogenic bacteria; thus, further comparison with other publications is not conceivable. However, there is one report about testing the extract of *P. odorata* against fish bacterial pathogenic by Najiah et al. (2011). Their study found that the methanolic and aqueous extract of *P. odorata* had a mild level of antibacterial effectiveness against *S. aureus* with an inhibition zone of 8.00 mm but no antibacterial activity against *E. coli*, *E. tarda*, *A. hydrophila*, *C. freundii*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *S. agalactiae*, and *Streptococcus aginosus*. Meanwhile, the present study showed that the ethanolic extract of the plant has significant inhibitory effects on the growth of *A. hydrophila*, *A. jandaei*, *A. sobria*, and *E. tarda*. This discrepancy could be attributed to the extraction process and solvent used, which affect the concentration of active components in extracts and species variation (Farahmandfar et al., 2019).

## CONCLUSION

Due to the rapid rise of antibiotic-resistant organisms, there is an ongoing need to explore new antimicrobial compounds, especially from natural sources. The present study's findings demonstrated the potential of Malaysian traditional vegetables in inhibiting the growth of fish pathogenic bacteria, albeit to varying degrees. Due to their high antibacterial activity, *P. odorata* and *G. atroviridis* extracts are considered to have significant potential for future use as antibacterial agents in aquaculture. Moreover, additional investigation is required to identify these plants' specific active chemical compounds and their impact on fish metabolism. Another effective method for implementing extracts should also be considered.

## ACKNOWLEDGEMENTS

This research was supported using the International Islamic University Malaysia (IIUM) Flagship Research Initiative Grant Scheme (IRF19-003-0003).

## REFERENCES

AL Ubeed, H. M. S., Bhuyan, D. J., Alsherbiny, M. A., Basu, M., & Vuong, Q. V. (2022). A comprehensive review on the techniques for extraction of bioactive compounds from medicinal Cannabis. *Molecules*, *27*(3), 604. https://doi.org/10.3390/molecules27030604

Aminullah, F., Malek, N. A. N. N., & Jemon, K. (2021). Antibacterial activity of silver nanoparticles synthesized from *Persicaria odorata* (L.) sojak leaves extract. In A. Taufiq, H. Susanto, H. Nur, M. Aziz, A. Suksuwan, C. S. Ng, K. B. Jemon, M. Amin, M. Diantoro, N. Mufti, N. A. N. N. Malek, I. C. Wang, Sunaryono., S. Zubaidah, Aulanni'am., I. Wibowo, & A. Y. Handaya (Eds.), *International Conference on Life Sciences and Technology* (Vol. 2353, No. 1, p. 030022). AIP Publishing. https://doi.org/10.1063/5.0052607

Anastasiou, T. I., Mandalakis, M., Krigas, N., Vézignol, T., Lazari, D., Katharios, P., Dailianis T., & Antonopoulou, E. (2020). Comparative evaluation of essential oils from medicinal-aromatic plants of Greece: Chemical composition, antioxidant capacity and antimicrobial activity against bacterial fish pathogens. *Molecules*, *25*(1), 148. https://doi.org/10.3390/molecules25010148

Azmi, N., Zulkurnain, E. I., Ramli, S., James, R. J., & Halim, H. (2021). The phytochemical and pharmacological properties of *Persicaria odorata*: A review. *Journal of Pharmaceutical Research International*, *33*(41B), 262-279. https://doi.org/10.9734/jpri/2021/v33i41b32366

Bacayo, M. F. D. C., Sajali, N. S., Choon, W. C., Fattepur, S., Nilugal, K. C., Khan, J., Asmani, F., & Yusuf, E. (2018). The study of the antibacterial activity of asam gelugor (*Garcinia atroviridis*) against methecillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumoniae* and *Klebseilla pneumoniae*. In *Proceeding of BROMO Conference* (pp. 118-123). SciTePress – Science and Technology Publications.

Bektaş, S., & Özdal, M. (2022). Antimicrobial activity of *Eucalyptus* (*Eucalyptus camaldulensis*) essential oil against fish pathogen bacterium, *Aeromonas caviae*. *Marine Science and Technology Bulletin*, *11*(4), 467-474. https://doi.org/10.33714/masteb.1184165

Bondad-Reantaso, M. G., MacKinnon, B., Karunasagar, I., Fridman, S., Alday-Sanz, V., Brun, E., Le Groumellec, M., Li, A., Surachetpong, W., Karunasagar, I., Hao, B., Dall'Occo, A., Urbani, R., & Caputo, A. (2023). Review of alternatives to antibiotic use in

aquaculture. *Reviews in Aquaculture*, *15*(4), 1421-1451. https://doi.org/10.1111/raq.12786

Clinical and Laboratory Standards Institute. (2018). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically* (11th ed.). CLSI.

Eruygur, N., Koçyiğit, U. M., Taslimi, P., Ataş, M., Tekin, M., & Gülçin, İ. (2019). Screening the *in vitro* antioxidant, antimicrobial, anticholinesterase, antidiabetic activities of endemic *Achillea cucullata* (Asteraceae) ethanol extract. *South African Journal of Botany*, *120*, 141-145. https://doi.org/10.1016/j.sajb.2018.04.001

Farahmandfar, R., Kenari, R. E., Asnaashari, M., Shahrampour, D., & Bakhshandeh, T. (2019). Bioactive compounds, antioxidant and antimicrobial activities of *Arum maculatum* leaves extracts as affected by various solvents and extraction methods. *Food Science and Nutrition*, *7*(2), 465-475. https://doi.org/10.1002/fsn3.815

Ghasemzadeh, A., Jaafar, H. Z. E., Bukhori, M. F. M., Rahmat, M. H., & Rahmat, A. (2018). Assessment and comparison of phytochemical constituents and biological activities of bitter bean (*Parkia speciosa* Hassk.) collected from different locations in Malaysia. *Chemistry Central Journal*, *12*, 12. https://doi.org/10.1186/s13065-018-0377-6

Guo, H., Chen, J., Yuan, X., Zhang, J., Wang, J., Yao, J., & Ge, H. (2023). The combined effect of a novel formula of herbal extracts on bacterial infection and immune response in *Micropterus salmoides*. *Frontiers in Microbiology*, *14*, 1185234. https://doi.org/10.3389/fmicb.2023.1185234

Hussain, I. A., Jeyasekaran, G., Shakila, R. J., Raj, K. T., & Jeevithan, E. (2014). Detection of hemolytic strains of *Aeromonas hydrophila* and *A. sobria* along with other *Aeromonas* spp. from fish and fishery products by multiplex PCR. *Journal of Food Science and Technology*, *51*, 401-407. https://doi.org/10.1007/s13197-013-1190-9

Junior, G. B., Bianchini, A. E., de Freitas Souza, C., Descovi, S. N., da Silva Fernandes, L., de Lima Silva, L., Cargnelutti, J. F., & Baldisserotto, B. (2022). The use of cinnamon essential oils in aquaculture: Antibacterial, anesthetic, growth-promoting, and antioxidant effects. *Fishes*, *7*(3), 133. https://doi.org/10.3390/fishes7030133

Junior, G. B., de Freitas Souza, C., Baldissera, M. D., Descovi, S. N., da Silveira, B. P., Tasca, C., Mourão, R. H. V., de Vargas, A. P. C., & Baldisserotto, B. (2019). Plant essential oils against bacteria isolated from fish: An *in vitro* screening and *in vivo* efficacy of *Lippia origanoides*. *Ciência Rural*, *49*(6), e20190064. https://doi.org/10.1590/0103-8478cr20190064

Klūga, A., Terentjeva, M., Vukovic, N. L., & Kačániová, M. (2021). Antimicrobial activity and chemical composition of essential oils against pathogenic microorganisms of freshwater fish. *Plants*, *10*(7), 1265. https://doi.org/10.3390/plants10071265

Maran, B. A. V., Iqbal, M., Gangadaran, P., Ahn, B.-C., Rao, P. V., & Shah, M. D. (2022). Hepatoprotective potential of Malaysian medicinal plants: A review on phytochemicals, oxidative stress, and antioxidant mechanisms. *Molecules*, *27*(5), 1533. https://doi.org/10.3390/molecules27051533

Mohd Noor, H. S., Abu Bakar, M. F., Abu Bakar, F. I., Ismail, N. A., Sanusi, S. B., & Mohamed, M. (2020). Phytochemical content and antioxidant activity of selected wild ulam/vegetables consumed by indigenous Jakun community in Taman Negara Johor Endau Rompin (TNJER), Malaysia. *Food Research*, *4*(1), 28-33. https://doi.org/10.26656/fr.2017.4(1).179

Mostafa, A. A., Al-Askar, A. A., Almaary, K. S., Dawoud, T. M., Sholkamy, E. N., & Bakri,

M. M. (2018). Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. *Saudi Journal of Biological Sciences*, *25*(2), 361-366. https://doi.org/10.1016/j.sjbs.2017.02.004

Najiah, M., Nadirah, M., Arief, Z., Zahrol, S., Tee, L. W., Ranzi, A. D., Amar, A.S, Laith, A. R., Mariam, M., Suzana, S. & Aida, R. J. (2011). Antibacterial activity of Malaysian edible herbs extracts on fish pathogenic bacteria. *Research Journal of Medicinal Plant*, *5*(6), 772-778. https://doi.org/10.3923/rjmp.2011.772.778

Nguyen, V. T., Nguyen, M. T., Nguyen, N. Q., & Truc, T. T. (2020). Phytochemical screening, antioxidant activities, total phenolics and flavonoids content of leaves from *Persicaria odorata* Polygonaceae. In *IOP Conference Series: Materials Science and Engineering* (Vol. 991, No. 1, p. 012029). IOP Publishing. https://doi.org/10.1088/1757-899X/991/1/012029

Niyomdecha, A., Wattanachant, C., Rattanawut, J., Plodpai, P., & Ngampongsai, W. (2022). Antibacterial activity of ethanol extract of asam gelugur (*Garcinia atroviridis*) fruits from Southern Thailand. *The Thai Journal of Pharmaceutical Sciences*, *46*(3), 346-352. https://doi.org/10.56808/3027-7922.2580

Okon, E. M., Okocha, R. C., Adesina, B. T., Ehigie, J. O., Alabi, O. O., Bolanle, A. M., Matekwe, N., Falana, B. M., Tiamiyu, A. M., Olatoye, I. O. & Adedeji, O. B. (2022). Antimicrobial resistance in fish and poultry: Public health implications for animal source food production in Nigeria, Egypt, and South Africa. *Frontiers in Antibiotics*, *1*, 1043302. https://doi.org/10.3389/frabi.2022.1043302

Pepi, M., & Focardi, S. (2021). Antibiotic-resistant bacteria in aquaculture and climate change: A challenge for health in the Mediterranean area. *International Journal of Environmental Research and Public Health*, *18*(11), 5723. https://doi.org/10.3390/ijerph18115723

Rahman, M. T., Nielsen, R. & Khan, M. A. (2022). Pond aquaculture performance over time: A perspective of small-scale extensive pond farming in Bangladesh. *Aquaculture Economics and Management*, *26*(2), 192-214. https://doi.org/10.1080/13657305.2021.1979122

Reang, J., Sharma, P. C., Thakur, V. K., & Majeed, J. (2021). Understanding the therapeutic potential of ascorbic acid in the battle to overcome cancer. *Biomolecules*, *11*(8), 1130. https://doi.org/10.3390/biom11081130

Řebíčková, K., Bajer, T., Šilha, D., Houdková, M., Ventura, K., & Bajerová, P. (2020). Chemical composition and determination of the antibacterial activity of essential oils in liquid and vapor phases extracted from two different southeast Asian herbs —*Houttuynia cordata* (Saururaceae) and *Persicaria odorata* (Polygonaceae). *Molecules*, *25*(10), 2432. https://doi.org/10.3390/molecules25102432

Ridzuan, P. M., Aini, H., Norazian., & Shah, A. (2013). Antibacterial and antifungal properties of *Persicaria odorata* leaf against pathogenic bacteria and fungi. *The Open Conference Proceedings Journal*, *4*, 222. https://doi.org/10.2174/2210289201304010222

Sakai, T., Yuasa, K., Sano, M., & Iida, T. (2009). Identification of *Edwardsiella ictaluri* and *E. tarda* by species-specific polymerase chain reaction targeted to the upstream region of the fimbrial gene. *Journal of Aquatic Animal Health*, *21*(2), 124-132. https://doi.org/10.1577/H08-061.1

Schobert, R., & Biersack, B. (2019). Chemical and biological aspects of garcinol and isogarcinol: Recent developments. *Chemistry and Biodiversity*, *16*(9), e1900366. https://doi.org/10.1002/cbdv.201900366

Shahid, M., Law, D., Azfaralariff, A., Mackeen, M. M., Chong, T. F., & Fazry, S. (2022). Phytochemicals and biological activities of *Garcinia atroviridis*:

A critical review. *Toxics*, *10*(11), 656. https://doi.org/10.3390/toxics10110656

Silva, N. C. C., & Fernandes Júnior, A. (2010). Biological properties of medicinal plants: A review of their antimicrobial activity. *Journal of Venomous Animals and Toxins Including Tropical Diseases*, *16*(3), 402-413. https://doi.org/10.1590/S1678-91992010000300006

Tan, W.-N., Khairuddean, M., Khaw, K.-Y., Murugaiyah, V., Yenn, T. W., Ring, L. C., & Ibrahim, D. (2019). Phytochemical screening and biological evaluations of *Garcinia atroviridis*. *Iranian Journal of Pharmaceutical Sciences*, *15*(2), 91-104. https://doi.org/10.22037/ijps.v15.40573

Tan, W.-N., Khairuddean, M., Wong, K.-C., Tong, W.-Y., & Ibrahim, D. (2016). Antioxidant compounds from the stem bark of *Garcinia atroviridis*. *Journal of Asian Natural Products Research*, *18*(8), 804-811. https://doi.org/10.1080/10286020.2016.1160071

Thongkham, E., Aiemsaard, J., & Kaenjampa, P. (2021). Antioxidant and antimicrobial properties of ethanolic extract of asam gelugor fruit (*Garcinia atroviridis*). *Burapha Science Journal*, *26*(2), 1293-1307.

Tkachenko, H., Pękala-Safińska, A., Buyun, L., & Kurhaluk, N. (2022). A comparative assessment of the antibacterial activity of extracts derived from leaves of various *Ficus* species (Moraceae) against fish pathogens. *Fisheries and Aquatic Life*, *30*(4), 217-231. https://doi.org/10.2478/aopf-2022-0021

Tkachenko, H., Pękala-Safińska, A., Buyun, L., Honcharenko, V., Prokopiv, A., & Kurhaluk, N. (2023). Antibacterial activity of extracts derived from leaves of *Ficus elastica* Roxb. ex Hornem. (Moraceae) and its cultivars against three *Aeromona*s spp. strains. *Agrobiodiversity for Improving Nutrition, Health and Life Quality*, *7*(1), 70-79.

Vaou, N., Stavropoulou, E., Voidarou, C., Tsigalou, C., & Bezirtzoglou, E. (2021). Towards advances in medicinal plant antimicrobial activity: A review study on challenges and future perspectives. *Microorganisms*, *9*(10), 2041. https://doi.org/10.3390/microorganisms9102041

Zhang, W., Zhao, J., Ma, Y., Li, J., & Chen, X. (2022). The effective components of herbal medicines used for prevention and control of fish diseases. *Fish and Shellfish Immunology*, *126*, 73-83. https://doi.org/10.1016/j.fsi.2022.05.036

Pertanika J. Trop. Agric. Sci. 47 (4): 1325 - 1341 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Effects of Treatments and Fermentation Time on Phenolic Compounds, Glycoalkaloid Contents, and Antioxidant Capacity of Industrial Potato Waste

**Muhammad Surajo Afaka[1,2], Iswan Budy Suyub[1], Frisco Nobilly[1] and Halimatun Yaakub[1]***

*[1]Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

*[2]Department of Animal Science, Faculty of Agriculture, Kaduna State University, 800283, Tafawa Balewa Way, PMB 2339, Kaduna, Nigeria*

## ABSTRACT

Potato processing plants generate waste in the form of peels, pulp, and rejects, which is estimated to be around 12–20 % of their total production volume. Potato peels, pulp, and unmarketable potatoes can be processed and incorporated into animal feed formulations. However, there is a limited information on phenolic compounds from industrial potato waste (IPW) subjected to short-term solid-state fermentation. Bioactive compounds could be improved via solid-state fermentation. *Lactiplantibacillus plantarum* (MW296876), *Saccharomyces cerevisiae* (MW296931), and *Aspergillus oryzae* (MW297015) were purposely selected to ferment IPW at 0, 24, 48, and 72 hr in a two-factor factorial design (treatment × fermentation time). The fermented products were analysed for phytochemical compounds such as total phenolic content (TPC), total flavonoid content (TFC), glycoalkaloid (GLA) content, and antioxidant capacity. The results revealed that the bioactive compounds, except phytic acid, had a significant interaction between treatment and fermentation time. Alpha solanine significantly ($p$<0.05) decreased while α chaconine increased ($p$<0.05) with fermentation time across all the treatments except in the control and *L. plantarum* treatment groups. IPW inoculated with *L. plantarum* significantly influenced the solubility of GLA compared to other treatment groups. Antioxidant capacity increased ($p$<0.05) across the fermentation time; at 48 hr of fermentation, *L. plantarum* had the highest ($p$<0.05)

ARTICLE INFO

*Article history:*
Received: 03 February 2024
Accepted: 27 March 2024
Published: 19 November 2024

DOI: https://doi.org/10.47836/pjtas.47.4.16

*E-mail addresses*:
surajo.mohammed@kasu.edu.ng (Muhammad Surajo Afaka)
iswan@upm.edu.my (Iswan Budy Suyub)
frisco@upm.edu.my (Frisco Nobilly)
hali@upm.edu.my (Halimatun Yaakub)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

antioxidant capacity than *S. cerevisiae* and *A. oryzae*. Among the three inocula, *L. plantarum* (MW296876) consistently increased TPC, antioxidant activity, and solubility of both GLA and tannin.

*Keywords*: Antioxidants, fermentation, glycoalkaloids, phytochemical compounds, potato waste

## INTRODUCTION

Potato (*Solanum tuberosum* L.) contains vitamins, minerals, proteins, dietary fibre, and phytochemical compounds such as phenolic acids, flavonoids, and carotenoids (Cebulak et al., 2022). Phytochemicals are the largest group of phenolic compounds that account for most antioxidant activity in plants or plant products. Phytochemicals in potatoes are bioactive compounds that have wide effects on animal well-being (Hellmann et al., 2021).

The concentration of phenolic components varied in different scientific reports; chlorogenic acid has always been reported as the most abundant (Valiñas et al., 2017). It was reported that chlorogenic acid represents 90% of the total phenolic compounds in potato peels (Hellmann et al., 2021; Naveed et al., 2018). Other important phenolic acids include caffeic, ferulic, gallic, and syringic acids. The variation in concentration of phenolic acids is related to genotypes, extraction, and analytical methods (Akyol et al., 2016). It was reported that phenolic acids and flavonoids are the most abundant phenolic compounds, which are present in both free and bound forms (Ru et al., 2019) and are located in the peels and adjoining tissues (Akyol et al., 2016; Yılmaz et al., 2017).

The presence of flavonoids influences the flavour and colour of fruits (Akyol et al., 2016). Some important flavonoids in potatoes include catechin, quercetin, anthocyanins, and kaempferol (Kim et al., 2019). In addition, some researchers observed the presence of rutin (Rodríguez-Martínez et al., 2021). Depending on the skin colour, the concentration of flavonoid, on a fresh-weight basis, ranges between 30 to 60 mg/100 g (Akyol et al., 2016). The higher content of flavonoids in red and purple potatoes is due to the higher content of anthocyanins (Frond et al., 2019).

In the past decades, phenolic compounds have been considered antinutrients; however, in recent times, they have been believed to be micronutrients with a high antioxidant capacity (Peluso, 2019). Antioxidants have wide bioactive properties, including prevention of oxidation in food materials, antimicrobial activities, and neutralising free radicals produced within the body (Hur et al., 2014).

Over 80 different steroidal glycoalkaloids (SGLAs) have been identified in potatoes, but α-solanine and α-chaconine are by far the most predominant (Kondamudi et al., 2017). However, concentrations of glycoalkaloids are higher in the potato peel than in the flesh (Ok & Şanlı, 2022). It was reported that GLA contents in potatoes are influenced by variety, postharvest storage conditions, and the type of fertiliser applied to soil (Ok & Şanlı, 2022). In general, GLA is heat stable and resistant to degradation via exposure to

boiling water, baking, frying, and microwave irradiation (Omayio et al., 2016). It was reported that the toxicity of α-chaconine is higher than α-solanine. However, both have a synergistic effect when present in the same tissue; the severity of their toxicity depends on their concentrations and ratio, which ranges from 1:2 to 1:7. It was observed that after peeling, slicing and washing out in water, α-solanine content decreased more than α-chaconine (Omayio et al., 2016).

Despite the fact that potatoes and their by-products contain GLA, previous studies show that it is being used in animal diets in the forms of fresh, dried, steamed, and ensiled (Ncobela et al., 2017). Similarly, it was reported that potatoes or their by-products could replace 10 to 40% of corn in beef cattle diets without any deleterious effects on animal growth, health, or meat quality (Paradhipta et al., 2020). Although GLAs are widely considered toxic substances, some recent studies have reported that they contain various health-promoting properties, which include a strong antioxidant capacity and anti-cancer activities (Hellmann et al., 2021; Wu et al., 2018).

Depending on the processing method and variety of potatoes, potato waste can range between 15 to 40% of the original fresh weight (Sepelev & Galoburda, 2015). However, local production of potatoes stands at 215,632.40 t in 2022, with a potential yield of 8.3 t/ha (Department of Agriculture [DOA], 2022). According to the Food and Agriculture Organization of the United Nations (FAO) (2023), Malaysia imported 217,636.81 t of potatoes in 2021 (FAO, 2023). The combined volumes of potatoes (local and import) could generate 64,99.38 to 173,307.68 t of waste (peels/skin/low grades and rejected potatoes) from processing plants, households, and restaurants. The higher composition of peels in the IPW virtually increased its phenolic contents than the tuber fraction. Nevertheless, few studies have been conducted on IPW as a potential source of bioactive compounds for ruminant animals, especially when subjected to microbial inoculation and short-term solid-state fermentation.

The objective of this study was to determine phenolic compounds, glycoalkaloid compounds, and antioxidant capacity of industrial potato waste fermented with *L. plantarum* (MW296876), *S. cerevisiae* (MW296931), and *A. oryzae* (MW297015). Although IPW could be stored fresh for a couple of months under controlled temperatures with good ventilation, such a process is economically impractical. Alternatively, IPW could be preserved longer by ensiling alone or in a mixture of straws or grasses. On the other hand, IPW could be processed by short-term fermentation via a solid-state fermentation method, which lasts for a couple of days (< 7 days). The major limitation of the longer ensiling method is the duration of time (≥ 21 days) before the substrate reaches a stable anaerobic condition. A short-term fermentation provides an opportunity to quickly improve the nutrient and phenolic content of feedstuffs as well as reduce antinutrient factors (ANF) within a few days (24-96 hr). Afterwards, the feedstuff

could be dried to ensure a longer shelf-life before feeding or incorporating it into a ruminant diet.

## MATERIALS AND METHODS

### Fermentation of Substrate

About 100 kg of IPW was collected from French Fries (Malaysia) Sdn. Bhd., a local potato processing company in Malaysia. The IPW was then oven-dried at 65°C, cooled at room temperature (~ 28°C), milled, sieved to pass 1.0 mm and preserved in a cold room (4°C) until required for solid-state fermentation. A short-term IPW (substrate) fermentation was carried out using a method described by Aruna et al. (2017). Hence, about 100 g dry matter (DM) of the sample (substrate) was placed in a 250 ml Erlenmeyer flask. The moisture content was then adjusted to 60% by adding 140 ml of water, and then the flask was covered with a thin layer of paraffin. Thus, eighty samples were prepared and divided into four treatment groups. The first treatment group (control) was not inoculated with any microbe. In contrast, the remaining three treatment groups were inoculated with *L. plantarum* (MW296876) at $1\times10^5$/g (Abdul Rahman et al., 2017), *S. cerevisiae* (MW296931) at $1\times10^5$/g (Abdul Rahman et al., 2017), and *A. oryzae* (MW297015) at about 1 $cm^2$ of corresponding fungus per 50 g substrate (Ramin et al., 2011). Isolates of the three inoculants were initially obtained from the Malaysian Agricultural Research and Development Institute (MARDI). After the inoculation, five replicates from each treatment were randomly subjected to fermentation times of 0, 24, 48, and 72 hr. The incubation temperature was maintained at 35°C inside the incubator (LM-450D, BIOBASE, China). Termination of fermentation was carried out by increasing the incubation temperature to 65°C until a constant weight of substrate was observed. The inoculated substrates were then carefully removed from the Erlenmeyer flask and preserved in a refrigerator until chemical analyses were required.

### Chemical Assays

Bioactive compounds were extracted from IPW using a method described by Ji et al. (2012). Following the procedure described by Ji et al. (2012), TPC was determined by the Folin-ciocalteu method, antioxidant activity was assayed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, and glycoalkaloid (GLA) content was also determined via high-performance liquid chromatography (HPLC). A simple ratio between solanine and chaconine was calculated by dividing chaconine concentration by solanine content. Furthermore, TFC was determined using the aluminium chloride ($AlCl_3$) method described by Sulaiman and Balachandran (2012). Phytic acid content (mg/g) was determined by the iron (III) chloride ($FeCl_3$) method described by Adeyemo and Orilude (2013). Tannin and carotenoid contents were analysed using the procedure described by Ru et al. (2019).

The extracts of fermented samples (Ji et al., 2012) were proceeded for GLA assay via HPLC (Ultimate 3000, Thermo

Fisher Scientific, USA) with ultraviolet to visible (UV-Vis) detection at 200 nm wavelength. Standard solutions containing both α-chaconine (PHL80075, Sigma-Aldrich, USA) and α-solanine (PHL80074, Sigma-Aldrich, USA) were prepared from stock solutions. A five-point standard curve was made for each compound with a linear range between 1 to 100 ppm (µg/ml). Glycoalkaloid compounds were separated by injecting 20 µl of the prepared sample across Siliachrome column (4.6 mm × 250 mm × 5 µm) (Siliachrome Plus C18, Canada) after the column temperature was adjusted to 25°C. A mixture (v/v) of acetonitrile (30%, Sigma-Aldrich, USA) and 0.05 M monobasic ammonium phosphate buffer (70%, ACS reagent, Sigma-Aldrich, USA) with pH 6.5 was used as a mobile phase at a flow rate of 1.0 ml/min. The GLA results were reported as mg per gram of dry sample.

### Research Design and Statistical Analysis

IPW collected from processing plants was fermented with zero inoculants, *L. plantarum* (MW296876), *S. cerevisiae* (MW296931), and *A. oryzae* (MW297015) for 0, 24, 48, and 72 hr. The experiment's layout was a two-factor factorial design (treatment × fermentation time), with fermentation time randomly allotted to the treatment in a completely randomised design (CRD).

Data obtained were subjected to analysis of variance (ANOVA) using a general linear model (GLM) of SAS software version 9.4 (SAS, 2011). The model of the analysis was as follows:

$$Y_{ijr} = \mu + t_i + d_j + (td)_{ij} + e_{ijr}.$$

where, $Y_{ijr}$ = dependent variable/observation; $\mu$ = overall population mean; $t_i$ = effect of treatment; $d_j$ = effect of fermentation time; $(td)_{ij}$ = effect of interaction between treatment and fermentation time; $e_{ijr}$ = random error/residual

Means were separated using Duncan's multiple range tests (Duncan, 1955).

## RESULTS

### Total Phenolic, Flavonoid, and Carotenoid Contents

There was significant ($p$<0.05) interaction between treatment and time of fermentation across all the treatments (Table 1). The TPC of fermented IPW shows an increased value with increasing fermentation time across the treatments. Both fermented IPW with *L. plantarum* and *A. oryzae* recorded the highest TPC at 72 hr of fermentation, while *S. cerevisiae* recorded the highest TPC at 48 hr. At 72 hr of fermentation, fermented IPW with *L. plantarum* had higher ($p$<0.05) TPC than another treatment group.

There was a significant ($p$<0.05) interaction between treatment and time of fermentation for fermented IPW with the three different inoculants. However, no significant ($p$>0.05) difference was observed in total flavonoid contents across the treatments. Fermented IPW with *S. cerevisiae* and *A. oryzae* inevitably showed a significant ($p$<0.05) difference across the fermentation time. In these two treatments, it was observed that total flavonoid content significantly ($p$<0.05) increased with the

Table 1
*Total phenolic, flavonoid, and carotenoid contents of fermented industrial potato waste with* Lactiplantibacillus plantarum, Saccharomyces cerevisiae, *and* Aspergillus oryzae *inoculants at different time*

| Parameters | Time (hr) | Treatments | | | | *p*-value | |
|---|---|---|---|---|---|---|---|
| | | Control | *Lactiplantibacillus plantarum* | *Saccharomyces cerevisiae* | *Aspergillus oryzae* | Treatment | Treatment*Time |
| Total phenolic content (mg/g) | 0 | $48.51\pm0.12^{d}$ | $48.23\pm0.06^{d}$ | $48.32\pm0.17^{d}$ | $48.32\pm0.19^{c}$ | 0.5811 | <.0001 |
| | 24 | $73.02\pm0.36^{cB}$ | $77.01\pm0.36^{cA}$ | $33.90\pm0.28^{cD}$ | $47.43\pm0.44^{cC}$ | <.0001 | <.0001 |
| | 48 | $90.16\pm0.33^{bA}$ | $82.74\pm0.48^{bC}$ | $86.50\pm1.05^{aB}$ | $59.78\pm0.57^{bD}$ | <.0001 | <.0001 |
| | 72 | $98.47\pm0.38^{aB}$ | $151.53\pm0.29^{aA}$ | $81.71\pm0.54^{bC}$ | $67.90\pm0.55^{aD}$ | <.0001 | <.0001 |
| | *p*-value | <.0001 | <.0001 | <.0001 | <.0001 | | |
| Total flavonoid content (mg QE/g) | 0 | 14.25±0.66 | 11.87±2.20 | $9.71\pm0.46^{b}$ | $12.56\pm2.20^{b}$ | 0.2889 | 0.0387 |
| | 24 | 16.24±0.79 | 13.48±1.35 | $12.18\pm1.63^{b}$ | $16.65\pm1.60^{ab}$ | 0.1018 | 0.0387 |
| | 48 | 17.30±1.70 | 14.28±1.55 | $17.87\pm1.38^{a}$ | $13.44\pm1.24^{b}$ | 0.1286 | 0.0387 |
| | 72 | 14.99±2.89 | 17.23±1.20 | $19.16\pm0.79^{a}$ | $19.42\pm0.99^{a}$ | 0.2585 | 0.0387 |
| | *p*-value | 0.6294 | 0.1670 | <.0001 | 0.0274 | | |
| Total carotenoid content (mg/kg DW) | 0 | $96.04\pm1.74^{a}$ | $96.04\pm1.74^{a}$ | $94.59\pm1.28^{a}$ | $97.01\pm1.67^{a}$ | 0.7721 | <.0001 |
| | 24 | $39.10\pm14.57^{bAB}$ | $65.15\pm6.53^{bA}$ | $7.72\pm1.28b^{C}$ | $23.65\pm8.78^{bBC}$ | 0.0118 | <.0001 |
| | 48 | $8.20\pm1.74^{cB}$ | $19.79\pm4.21^{cA}$ | $10.62\pm0.96^{bB}$ | $25.58\pm1.28^{bA}$ | 0.0031 | <.0001 |
| | 72 | $9.65\pm1.28^{c}$ | $10.62\pm0.48^{c}$ | $10.62\pm2.94^{b}$ | $8.21\pm0.97^{c}$ | 0.7206 | <.0001 |
| | *p*-value | <.0001 | <.0001 | <.0001 | <.0001 | | |

*Note.* $^{A, B, C, D}$ mean values with different superscripts within the same row were significantly different at $p<0.05$; $^{a, b, c}$ mean values with different superscripts within the same column were significantly different at $p<0.05$; DW = Dry weight

fermentation time; the highest values were recorded between 48 and 72 hr of fermentation.

The results on total carotenoid content (TCC) show a significant ($p$<0.05) interaction between treatment and fermentation time. No difference ($p$>0.05) in carotenoid content was observed between treatments after 48 hr of fermentation. It was observed that the carotenoid content of fermented IPW decreased significantly ($p$<0.05) with fermentation time across all the treatments. At 72 hr of fermentation, the carotenoid content was similar ($p$>0.05) across all the treatments.

**Tannin Content of Fermented**

The tannin content presented in Table 2 shows a significant ($p$<0.05) interaction between treatment and fermentation time. It was also observed that the tannin content of all fermented IPW increased ($p$<0.05) over the fermentation time. At 24 hr of fermentation, fermented IPW with *S. cerevisiae* produced the highest ($p$<0.05) tannin content than the other treatments and the control. However, at 48 hr, the tannin content of fermented IPW with *S. cerevisiae* was at par ($p$>0.05) with *L. plantarum* and the control treatments, which were statistically higher ($p$<0.05) than *A. oryzae*.

**Glycoalkaloid Content**

The concentration of α-solanine (Table 2) decreased ($p$<0.05) linearly with fermentation time across all treatments except for the control. Fermented IPW with *L. plantarum* recorded a significant ($p$<0.05) increase in α-solanine content. Invariably, at 72 hr of fermentation, fermented IPW with *S. cerevisiae* and *A. oryzae* inoculants recorded significantly ($p$<0.05) least α-solanine content.

The chaconine content ($p$<0.05) significantly increased with the fermentation time across all treatments. A concentration of 0.138 mg/g chaconine was recorded for unfermented IPW. At 72 hr of fermentation, fermented IPW with *L. plantarum* recorded ($p$<0.05) higher concentration (0.479 mg/g) than all other treatments. However, 0.450 mg/g recorded for the control was significantly ($p$<0.05) higher than 0.254 and 0.298 mg/g observed for fermented IPW with *S. cerevisiae* and *A. oryzae*, respectively.

Across all the treatments, except for fermented IPW with *A. oryzae*, which increased to 1:5 at 24 hr of fermentation, the ratios of α solanine to α chaconine (Table 3) varied from 1:2 at 0 hr to 1:3 at 24 and 48 hr. At 72 hr, the concentration of α-chaconine in the control and fermented IPW with *L. plantarum* decreased to 1:1. However, fermented IPW with *S. cerevisiae* and *A. oryzae* recorded an increase in α chaconine content with a ratio of 1:4.

**Phytic Acid Content**

The result on phytic acid content (Table 4) shows no significant ($p$>0.05) interaction between treatments and time of fermentation. No significant ($p$>0.05) difference was observed across the treatments except at 48 hr, where the control recorded the highest concentration of phytic acid than

Table 2
*Tannin, solanine, and chaconine contents of fermented industrial potato waste with* Lactiplantibacillus plantarum, Saccharomyces cerevisiae, *and* Aspergillus oryzae *inoculants at different time*

| Parameters | Time (hr) | Treatments | | | | p-value | |
|---|---|---|---|---|---|---|---|
| | | Control | *Lactiplantibacillus plantarum* | *Saccharomyces cerevisiae* | *Aspergillus oryzae* | Treatment | Treatment*Time |
| Tannin (mg/g) | 0 | 0.47±0.02 | $0.47\pm0.01^{c}$ | $0.47\pm0.02^{c}$ | $0.47\pm0.02^{a}$ | 0.9996 | <.0001 |
| | 24 | $0.53\pm0.02^{C}$ | $0.75\pm0.02^{bB}$ | $1.06\pm0.03^{aA}$ | $0.15\pm0.01^{cD}$ | <.0001 | <.0001 |
| | 48 | $0.47\pm0.03^{A}$ | $0.49\pm0.02^{cA}$ | $0.43\pm0.01^{cA}$ | $0.27\pm0.00^{bB}$ | <.0001 | <.0001 |
| | 72 | $0.47\pm0.02^{C}$ | $1.20\pm0.01^{aA}$ | $0.83\pm0.02^{bB}$ | $0.41\pm0.04^{aC}$ | <.0001 | <.0001 |
| | *p*-value | 0.1858 | <.0001 | <.0001 | <.0001 | | |
| α-solanine (mg/g) | 0 | $0.094^{b}$ | $0.094^{b}$ | $0.094^{a}$ | $0.094^{a}$ | 1.0000 | <.0001 |
| | 24 | $0.080^{dA}$ | $0.079^{dA}$ | $0.062^{dB}$ | $0.051^{dC}$ | <.0001 | <.0001 |
| | 48 | $0.085^{cA}$ | $0.072^{cC}$ | $0.067^{bD}$ | $0.079^{bB}$ | <.0001 | <.0001 |
| | 72 | $0.373^{aB}$ | $0.382^{aA}$ | $0.065^{cD}$ | $0.074^{cC}$ | <.0001 | <.0001 |
| | *p*-value | <.0001 | <.0001 | <.0001 | <.0001 | | |
| α-chaconine (mg/g) | 0 | $0.138^{d}$ | $0.138^{d}$ | $0.138^{d}$ | $0.138^{d}$ | 1.0000 | <.0001 |
| | 24 | $0.263^{cA}$ | $0.239^{cB}$ | $0.159^{cD}$ | $0.237^{cC}$ | <.0001 | <.0001 |
| | 48 | $0.211^{bD}$ | $0.242^{bB}$ | $0.227^{bC}$ | $0.249^{bA}$ | <.0001 | <.0001 |
| | 72 | $0.451^{aB}$ | $0.481^{aA}$ | $0.255^{aD}$ | $0.299^{aC}$ | <.0001 | <.0001 |
| | *p*-value | <.0001 | <.0001 | <.0001 | <.0001 | | |

*Note.* $^{A, B, C, D}$ mean values with different superscripts within the same row were significantly different at $p<0.05$; $^{a, b, c, d}$ mean values with different superscripts along the same column were different at $p<0.05$; NB = The SEM of α-solanine and α-chaconine in all the observations was ≤ 0.00057735

Table 3
*Solanine to chaconine ratio of fermented industrial potato waste with* Lactiplantibacillus plantarum, Saccharomyces cerevisiae, *and* Aspergillus oryzae *inoculants at different time*

| Treatment | Fermentation time (hr) | | | |
|---|---|---|---|---|
| | 0 | 24 | 48 | 72 |
| Control | 1:2 | 1:3 | 1:3 | 1:1 |
| *Lactiplantibacillus plantarum* | 1:2 | 1:3 | 1:3 | 1:1 |
| *Saccharomyces cerevisiae* | 1:2 | 1:3 | 1:3 | 1:4 |
| *Aspergillus oryzae* | 1:2 | 1:5 | 1:3 | 1:4 |

Table 4
*Phytic acid contents of fermented industrial potato waste with* Lactiplantibacillus plantarum, Saccharomyces cerevisiae, *and* Aspergillus oryzae *inoculants at different time*

| Time (hr) | Treatment | | | | p-value | |
|---|---|---|---|---|---|---|
| | Control | *Lactiplantibacillus plantarum* | *Saccharomyces cerevisiae* | *Aspergillus oryzae* | Treatment | Treatment*Time |
| 0 | 1.95±0.11 | 2.05±0.11$^{a}$ | 2.06±0.12$^{a}$ | 2.14±0.12$^{a}$ | 0.7000 | 0.1415 |
| 24 | 1.85±0.10 | 1.56±0.09$^{b}$ | 1.75±0.10$^{ab}$ | 1.68±0.09$^{b}$ | 0.2679 | 0.1415 |
| 48 | 1.82±0.10$^{A}$ | 1.47±0.08$^{bB}$ | 1.65±0.09$^{bAB}$ | 1.48±0.08$^{bcB}$ | 0.0419 | 0.1415 |
| 72 | 1.75±0.10$^{A}$ | 1.39±0.08$^{bB}$ | 1.56±0.09$^{bAB}$ | 1.30±0.07$^{cB}$ | 0.0231 | 0.1415 |
| *p*-value | 0.6178 | 0.0036 | 0.0310 | 0.0012 | | |

*Note*. $^{A, B,}$ mean values with different superscripts within the same row were significantly different at $p<0.05$; $^{a, b, c,}$ mean values with different superscripts along the same column were different at $p<0.05$

Table 5
*Antioxidant capacity of fermented industrial potato waste with* Lactiplantibacillus plantarum, Saccharomyces cerevisiae, *and* Aspergillus oryzae *inoculants at different time*

| Fermentation time (hr) | Treatments | | | | *p*-value | |
|---|---|---|---|---|---|---|
| | Control | *Lactiplantibacillus plantarum* | *Saccharomyces cerevisiae* | *Aspergillus oryzae* | Treatment | Treatment *Time |
| 0 | 33.90±0.12$^{c}$ | 33.94±0.09$^{c}$ | 33.94±0.09$^{b}$ | 33.99±0.10$^{c}$ | 0.9406 | <.0001 |
| 24 | 33.66±0.36$^{cC}$ | 42.07±0.11$^{aA}$ | 38.81±0.20$^{aB}$ | 42.28±0.41$^{aA}$ | <.0001 | <.0001 |
| 48 | 40.31±0.10$^{aB}$ | 41.39±0.086$^{bA}$ | 38.79±0.15$^{aC}$ | 40.04±0.14$^{bB}$ | <.0001 | <.0001 |
| 72 | 35.94±0.09$^{bA}$ | 31.51±0.08$^{dC}$ | 34.39±0.18$^{bB}$ | 27.02±0.05$^{dD}$ | <.0001 | <.0001 |
| *p*-value | <.0001 | <.0001 | <.0001 | <.0001 | | |

*Note*. $^{A, B, C, D}$ mean values with different superscripts within the same row were significantly different at $p<0.05$; $^{a, b, c, d}$ mean values with different superscripts along the same column were different at $p<0.05$

the other three treatments that were similar. Invariably, it was observed that there was a significant ($p$<0.05) difference across the fermentation time except for the control. It was also observed that the phytic acid contents decreased linearly from 0 to 72 hr of fermentation time. At 72 hr fermentation time, *A. oryzae* recorded the least ($p$<0.05) phytic acid content.

### Antioxidant Capacity

The antioxidant capacity (Trolox equivalents mg/g) of fermented IPW increased significantly ($p$<0.05) across the time of fermentation until 48 hr (Table 5). Similarly, at 24 hr of fermentation, fermented IPW with *L. plantarum* and *A. oryzae* recorded a similar ($p$>0.05) concentration of the antioxidant, which was higher ($p$<0.05) than the concentration observed in fermented IPW with *S. cerevisiae*. But at 48 hr, fermented IPW with *L. plantarum* recorded the highest ($p$<0.05) concentration of antioxidants than both fermented IPW with *S. cerevisiae* and *A. oryzae* inoculants.

## DISCUSSION

Plants produced phenolic compounds during phenylpropanoid biosynthesis via shikimate pathways (Nkhata et al., 2018). It was observed that plants can convert synthesised phenolic components such as ferulic acid and caffeic acids into other related compounds like lignin, tannins, and flavonoids. Notwithstanding, common phenolic acids in potato peels are chlorogenic acid, chlorogenic acid isomer, and caffeic acid (Friedman et al., 2017). The findings of this study show that the fermentation of IPW corroborated an earlier report that the fermentation process could result in an increase or decrease in phytochemical contents (Nkhata et al., 2018). The concentration of TPC recorded in the present work was within the range of earlier reports on different potato varieties (Ru et al., 2019). However, our result was higher than the values recorded in twenty potato clones (Ji et al., 2012) but lower than the concentrations recorded for both peeled and unpeeled potatoes (Ah-Hen et al., 2012). Variation in TPC has been attributed to many factors, including variety, skin colour, and tissue (tuber, flesh, and peel). Similarly, the increase in TPC observed in the present study could be related to the fact that during fermentation, phenolic compounds that are bound to proteins and minerals are released (Nkhata et al., 2018).

Flavonoids are a common group of phenolic compounds that influence fruits and vegetables' flavour and colour. Our study only analysed total flavonoid content, which should be composed of six significant subclasses: flavones, flavanols, flavanones, flavan-3-ols, anthocyanidins, and isoflavones. The present work recorded increased total flavonoid content over fermentation time, especially in treatments inoculated with *S. cerevisiae* and *A. oryzae*. It was also observed that inoculating industrial potato waste with *L. plantarum*, *S. cerevisiae*, and *A. oryzae* had no significant influence on total flavonoid content. The total flavonoid concentration recorded in our study was higher than the values

reported for organic and non-organic potato peel powders (Friedman et al., 2017). The variations observed from different reports on TFC could be related to various potato processing methods and analytical processes.

Carotenoids are important antioxidants that promote health, such as enhancing the immune system. It was discovered in the present work that the fermentation time and type of inoculation influenced the degradation of carotenoids. The work also revealed that *S. cerevisiae* and *A. oryzae* recorded higher degradation of total carotenoid content than the control and *L. plantarum* treatment groups. Similarly, it was observed that a high rate of degradation of total carotenoid content (mg/kg) occurred within 24 hr of fermentation. The degradation of total carotenoid content observed in our work corroborated an earlier finding that depending on the fermentation time, fermentation of high-carotenoid maize resulted in a significant loss of carotene (Ortiz et al., 2018). The values recorded in this study for total carotenoid content were higher but consistent with the fact that potato skin contains higher carotenoid content than flesh (Valcarcel et al., 2015). Among the various mechanisms proposed to explain the degradation of carotenoid content during fermentation is the disruption of the food matrix by endogenous enzymes and microorganisms, leading to the concentration of calcium, which might also enhance the saponification of free fatty acids (Nkhata et al., 2018). The most abundant carotenoids are α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene. In recent studies, fermentation either increased or decreased carotene content. The increase in carotenes during fermentation could be related to structural changes induced by fermentation, which increases the ability to extract carotenoids (Kiczorowski et al., 2022). However, loss of carotene during fermentation could also be related to the type of inoculum, time of fermentation, and oxidation (Zong et al., 2023). Therefore, differences in carotenoid content in different forms and morphological parts in potatoes could be associated with factors affecting carotenoid levels, such as genotype, climate, growing conditions, storage, and cooking processes (Lachman & Kotikova, 2016).

The effect of fermentation on tannin content (mg/g) was not consistent over time. Our result did not show a linear reduction of tannin due to fermentation time; however, a recent report on the fermentation of green coffee beans observed a significant reduction in tannin concentration (Haile & Kang, 2019). Reduction in tannin content during fermentation was previously recorded in cassava leaves (Hawashi et al., 2019). The tannin content recorded in this study was not higher than the values recorded for two potato cultivars (Taie et al., 2015). Our results showed that inoculation of IPW with the experimental microorganisms influenced tannin extraction from substrates rather than breaking down tannin into simple carbohydrate compounds. The absence of a reduction in tannin content in our results could be related to the fact that the present work recorded an increase in total flavonoid

content. Incidentally, condensed tannins contain flavonoids (flavan 3-ol or flavan 3, 4-diol) without a sugar core. In contrast, hydrolysable tannins comprise gallic acids with a sugar (mainly glucose) core (Das et al., 2020). Hence, an increase in flavonoid content due to microbial fermentation is likely to affect a reduction in tannin content during fermentation.

Our results on the phytic acid content of fermented IPW were not affected by inoculation but significantly influenced by the fermentation time. Phytic acid is a storage form of phosphorous in seeds and tubers; however, phytic acid is converted to phytate when it is bound to several mineral elements such as Ca, Fe, Zn, Mo, and Mg (Cominelli et al., 2020). The reduction of phytic acid during fermentation has been well-established (Kareem et al., 2017). Reduction of phytic acid could be related to the activities of phytase secreted by fermenting microorganisms (Nkhata et al., 2018). The values observed for phytic acid contents in fermented IPW were within a lower range of 3-23 mg/100 g fresh weight; a range of 3.39-61.34 mg/100 g fresh weight was reported for Indian potato cultivars (Joshi et al., 2021).

The results of glycoalkaloid content in fermented industrial potato waste showed that the fermentation time and inoculation of IPW influenced the concentration of GLA. It was also observed that the ratio between α-solanine and α-chaconine was affected by the fermentation time and inoculation. The range of glycoalkaloid concentrations recorded in the present study was lower by far compared to values observed in peels of some selected potato cultivars (Taie et al., 2015), but within a range of values reported for twenty potato clones (Ji et al., 2012), and mashed Idaho variety of potato (Kondamudi et al., 2017).

The concentration of α-solanine was consistently lower than α-chaconine. Thus, the ratios of α-solanine to α-chaconine conformed with the report of a 1:2 to 1:7 range of ratios between the two glycoalkaloids (Omayio et al., 2016). Calculation of ratios between α-solanine and α-chaconine is important because it was observed that α-chaconine has about five times more bioactive function than α-solanine (Friedman et al., 2017). Variations in GLA contents in different works could be due to differences in potato variety, storage and processing methods (Kondamudi et al., 2017). Recent work reported an increase in glycoalkaloid content due to a decrease in the moisture content of potato vine silage (Juanjuan et al., 2019). However, the report on potato vine silage also observed a reduction of glycoalkaloid content from fresh potato vine. In contrast, our findings show that α-chaconine content increased with fermentation time. This study recorded a sharp increase in both α-solanine and α-chaconine in the control and treatment groups fermented with *L. plantarum* at 72 hr of fermentation. An increase in GLA content was likely caused by the ability to extract GLA from solvent (methanol) and its solubility. It is thought that alcoholic solvents can break down food matrix and efficiently penetrate cell membranes, thus

permitting the extraction of a high amount of organic extracts (Nazarni et al., 2016).

Microbial enzymes such as β-glucosidase and lipase produced during fermentation can hydrolyse glucosides and break down plant cell walls to release organic compounds. In particular, *L. plantarum* has a strong glucosidase activity (Nkhata et al., 2018). Hence, apart from the effect of solvent and solubility of GLA, the sharp rise in glycoalkaloid concentration in the treatment group inoculated with *L. plantarum* could be ascribed to the ability of the lactic acid bacteria therein to produce β-glucosidase which can break down food matrix to release more GLA (Juanjuan et al., 2019).

The present study demonstrated that the antioxidant capacity of IPW fermented with *L. plantarum*, *S. cerevisiae*, and *A. oryzae* increased with the fermentation time. It was discovered that IPW inoculated with *L. plantarum* recorded the highest antioxidant capacity. Recently, potatoes have received special attention because of their high antioxidant activity (Ah-Hen et al., 2012). The antioxidant capacity (Trolox equivalents mg/g) observed in the present study was higher than values recorded for twenty potato extracts (peels, tuber, and granules). However, it corroborated that potato peels possess higher DPPH radical scavenging activity than any other potato part or form (Ji et al., 2012). It has been reported that the skin colour of potatoes influenced its antioxidant capacity owing to its higher level of phenolic content (Ji et al., 2012). In addition, our result confirmed that fermentation improves antioxidant activity by increasing the release of phenolic compounds, which act as reducing agents or hydrogen donors (Hur et al., 2014). However, the antioxidant activity could not be predicted based on total phenolic content alone because other compounds in the extract may have contributed to the overall antioxidant capacity. Besides, other factors such as species of microorganisms, pH, temperature, solvent, fermentation time, the water content of the substrate types, and aerobic or anaerobic conditions may influence antioxidant capacity (Hur et al., 2014). Therefore, the higher antioxidant capacity (Trolox equivalents mg/g) recorded in IPW inoculated with *L. plantarum* at just 24 hr of fermentation agreed with an observation that substrate fermented with lactic acid bacteria produced higher antioxidant activity than substrate fermented with *S. cerevisiae* (Hur et al., 2014; Zhao & Shah, 2014). The superiority of *L. plantarum* over the two inocula used in the study was probably due to its ability to produce β-glucosidase that has high enzymatic hydrolysis over polymerised phenolic compounds during fermentation.

## CONCLUSION

A short-term fermentation of industrial potato waste with *L. plantarum* (MW296876), *S. cerevisiae* (MW296931), and *A. oryzae* (MW297015) shows changes in bioactive compounds during fermentation. Apart from the effect of fermentation time, the inocula used increased antioxidant activity and concentrations of phenolic and glycoalkaloid compounds. The concentrations of

glycoalkaloids (α-solanine and α-chaconine) and their ratios were increased by both the fermentation time and treatments (inoculation with microorganisms). Both fermentation time and treatments were responsible for the increase in the antioxidant capacity of industrial potato waste. Although the three microorganisms used in the study increased the concentration of different bioactive compounds, glycoalkaloids, and antioxidant activity, *L. plantarum* was therefore recommended because it was consistent in increasing total phenolic compounds, antioxidant capacity, and extraction yield of tannin, α-solanine, and α-chaconine. A future study on the nutritional implication of incorporating fermented industrial potato waste into a ruminant diet is recommended.

## ACKNOWLEDGEMENTS

The authors wish to acknowledge the technical assistance and laboratory facilities of the Department of Animal Science, Universiti Putra Malaysia.

## REFERENCES

Abdul Rahman, N., Abd Halim, M. R., Mahawi, N., Hasnudin, H., Al-Obaidi, J. R., & Abdullah, N. (2017). Determination of the use of *Lactobacillus plantarum* and *Propionibacterium freudenreichii* application on fermentation profile and chemical composition of corn silage. *BioMed Research International*, *2017*, 2038062. https://doi.org/10.1155/2017/2038062

Adeyemo, S. M., & Onilude, A. A. (2013). Enzymatic reduction of anti-nutritional factors in fermenting soybeans by *Lactobacillus plantarum* isolates from fermenting cereals. *Nigerian Food Journal*, *31*(2), 84–90. https://doi.org/10.1016/S0189-7241(15)30080-1

Ah-Hen, K., Fuenzalida, C., Hess, S., Contreras, A., Vega-Gálvez, A., & Lemus-Mondaca, R. (2012). Antioxidant capacity and total phenolic compounds of twelve selected potato landrace clones grown in southern Chile. *Chilean Journal of Agricultural Research*, *72*(1), 3–9. https://doi.org/10.4067/s0718-58392012000100001

Akyol, H., Riciputi, Y., Capanoglu, E., Caboni, M. F., & Verardo, V. (2016). Phenolic compounds in the potato and its byproducts: An overview. *International Journal of Molecular Sciences*, *17*(6), 835. https://doi.org/10.3390/ijms17060835

Aruna, T. E., Aworh, O. C., Raji, A. O., & Olagunju, A. I. (2017). Protein enrichment of yam peels by fermentation with *Saccharomyces cerevisiae* (BY4743). *Annals of Agricultural Sciences*, *62*(1), 33-37. https://doi.org/10.1016/j.aoas.2017.01.002

Cebulak, T., Krochmal-Marczak, B., Stryjecka, M., Krzysztofik, B., Sawicka, B., Danilčenko, H., & Jarienė, E. (2022). Phenolic acid content and antioxidant properties of edible potato (*Solanum tuberosum* L.) with various tuber flesh colours. *Foods*, *12*(1), 100. https://doi.org/10.3390/foods12010100

Cominelli, E., Pilu, R., & Sparvoli, F. (2020). Phytic acid and transporters: What can we learn from *low phytic acid* mutants? *Plants*, *9*(1), 69. https://doi.org/10.3390/plants9010069

Das, A. K., Islam, M. N., Faruk, M. O., Ashaduzzaman, M., & Dungani, R. (2020). Review on tannins: Extraction processes, applications and possibilities. *South African Journal of Botany*, *135*, 58–70. https://doi.org/10.1016/j.sajb.2020.08.008

Department of Agriculture. (2022). *Statistik tanaman sayur-sayuran dan tanaman kontan* [Vegetable and cash crop statistic]. DOA. https://www.doa.

gov.my/doa/resources/aktiviti_sumber/sumber_awam/maklumat_pertanian/perangkaan_tanaman/statistik_tanaman_sayur_tanaman_kontan_2022.pdf

Duncan, D. B. (1955). Multiple range and multiple *F* tests. *Biometrics*, *11*(1), 1-42. https://doi.org/10.2307/3001478

Food and Agriculture Organization of the United Nations. (2023). *Crops and livestock products*. FAO. https://www.fao.org/faostat/en/#data/QCL/visualize

Friedman, M., Kozukue, N., Kim, H.-J., Choi, S.-H., & Mizuno, M. (2017). Glycoalkaloid, phenolic, and flavonoid content and antioxidative activities of conventional nonorganic and organic potato peel powders from commercial gold, red, and Russet potatoes. *Journal of Food Composition and Analysis*, *62*, 69–75. https://doi.org/10.1016/j.jfca.2017.04.019

Frond, A. D., Iuhas, C. I., Stirbu, I., Leopold, L., Socaci, S., Andreea, S., Ayvaz, H., Andreea, S., Mihai, S., Diaconeasa, Z., & Carmen, S. (2019). Phytochemical characterization of five edible purple-reddish vegetables: Anthocyanins, flavonoids, and phenolic acid derivatives. *Molecules*, *24*(8), 1536. https://doi.org/10.3390/molecules24081536

Haile, M., & Kang, W. H. (2019). Antioxidant activity, total polyphenol, flavonoid and tannin contents of fermented green coffee beans with selected yeasts. *Fermentation*, *5*(1), 29. https://doi.org/10.3390/fermentation5010029

Hawashi, M., Altway, A., Widjaja, T., & Gunawan, S. (2019). Optimization of process conditions for tannin content reduction in cassava leaves during solid state fermentation using *Saccharomyces cerevisiae*. *Heliyon*, 5(8), e02298. https://doi.org/10.1016/j.heliyon.2019.e02298

Hellmann, H., Goyer, A., & Navarre, D. A. (2021). Antioxidants in potatoes: A functional view on one of the major food crops worldwide. *Molecules*, *26*(9), 2446. https://doi.org/10.3390/molecules26092446

Hur, S. J., Lee, S. Y., Kim, Y.-C., Choi, I., & Kim, G.-B. (2014). Effect of fermentation on the antioxidant activity in plant-based foods. *Food Chemistry*, *160*, 346–356. https://doi.org/10.1016/j.foodchem.2014.03.112

Ji, X., Rivers, L., Zielinski, Z., Xu, M., MacDougall, E., Stephen, J., Zhang, S., Wang, Y., Chapman, R. G., Keddy, P., Robertson, G. S., Kirby, C. W., Embleton, J., Worrall, K., Murphy, A., De Koeyer, D., Tai, H., Yu, L., Charter, E., & Zhang, J. (2012). Quantitative analysis of phenolic components and glycoalkaloids from 20 potato clones and *in vitro* evaluation of antioxidant, cholesterol uptake, and neuroprotective activities. *Food Chemistry*, *133*(4), 1177–1187. https://doi.org/10.1016/j.foodchem.2011.08.065

Joshi, A., Kaundal, B., Raigond, P., Singh, B., Sethi, S., Bhowmik, A., & Kumar, R. (2021). Low-volume procedure to determine phytate and ascorbic acid in potatoes: Standardization and analysis of Indian cultivars. *Journal of Food Composition and Analysis*, *102*, 103998. https://doi.org/10.1016/j.jfca.2021.103998

Juanjuan, Z., Wei, W., Aiqiong, Q., Samten., & Tenzin-tarchen., & Bin, L. (2019). Effects of moisture content and additives on the fermentation quality and degradation of glycoalkaloids in potato (*Solanum tuberosum*) vine silage in Tibet. *American Journal of Agriculture and Forestry*, *7*(1), 1-9. https://doi.org/10.11648/j.ajaf.20190701.11

Kareem, K. A., Ojokoh, A., & Baba, J. (2017). The effects of fermentation on the nutritional and anti-nutritional constituents of Irish potato peels. *Annals. Food Science and Technology*, *18*(4), 680–685.

Kiczorowski, P., Kiczorowska, B., Samolińska, W., Szmigielski, M., & Winiarska-Mieczan, A. (2022). Effect of fermentation of chosen

vegetables on the nutrient, mineral, and bio component profile in human and animal nutrition. *Scientific Reports*, *12*,13422. https://doi.org/10.1038/s41598-022-17782-z

Kim, J., Soh, S. Y., Bae, H., & Nam, S.-Y. (2019). Antioxidant and phenolic contents in potatoes (*Solanum tuberosum* L.) and micropropagated potatoes. *Applied Biological Chemistry*, *62*, 17. https://doi.org/10.1186/s13765-019-0422-8

Kondamudi, N., Smith, J. K., & McDougal, O. M. (2017). Determination of glycoalkaloids in potatoes and potato products by microwave assisted extraction. *American Journal of Potato Research*, *94*, 153–159. https://doi.org/10.1007/s12230-016-9558-9

Lachman, J., Hamouz, K., Orsák, M., & Kotíková, Z. (2016). Carotenoids in potato – A short overview. *Plant, Soil and Environment*, *62*(10), 474–481.

Naveed, M., Hejazi, V., Abbas, M., Kamboh, A. A., Khan, G. J., Shumzaid, M., Ahmad, F., Babazadeh, D., FangFang, X., Modarresi-Ghazani, F., WenHua, L., & XiaoHui, Z. (2018). Chlorogenic acid (CGA): A pharmacological review and call for further research. *Biomedicine and Pharmacotherapy*, *97*, 67–74. https://doi.org/10.1016/J.BIOPHA.2017.10.064

Nazarni, R., Purnama, D., Umar, S., & Eni, H. (2016). The effect of fermentation on total phenolic, flavonoid and tannin content and its relation to antibacterial activity in jaruk tigarun (*Crataeva nurvala*, Buch HAM). *International Food Research Journal*, *23*(1), 309–315.

Ncobela, C. N., Kanengoni, A. T., Hlatini, V. A., Thomas, R. S., & Chimonyo, M. (2017). A review of the utility of potato by-products as a feed resource for smallholder pig production. *Animal Feed Science and Technology*, *227*, 107–117. https://doi.org/10.1016/J.ANIFEEDSCI.2017.02.008

Nkhata, S. G., Ayua, E., Kamau, E. H., & Shingiro, J.-B. (2018). Fermentation and germination improve nutritional value of cereals and legumes through activation of endogenous enzymes. *Food Science and Nutrition*, *6*(8), 2446–2458. https://doi.org/10.1002/fsn3.846

Ok, F. Z., & Şanlı, A. (2022). Potato glykoalkaloids: Properties and biological activities. *Atatürk University Journal of Agricultural Faculty*, *53*(1), 88-96.

Omayio, D. G., Abong, G. O., & Okoth, M. W. (2016). A review of occurrence of glycoalkaloids in potato and potato products. *Current Research in Nutrition and Food Science Journal*, *4*(3), 195–202. https://doi.org/10.12944/CRNFSJ.4.3.05

Ortiz, D., Nkhata, S., Buechler, A., Rocheford, T., & Ferruzzi, M. G. (2018). Nutritional changes during biofortified maize fermentation (steeping) for ogi production. *The FASEB Journal*, *31*(S1), 32.4. https://doi.org/10.1096/FASEBJ.31.1_supplement.32.4

Paradhipta, D. H. V., Lee, H.-J., Joo, Y.-H., Lee, S.-S., Kang, D.-H., Chung, K.-Y., & Kim, S.-C. (2020). Effects of potato by-products containing glycoalkaloid on rumen fermentation characteristics. *Journal of Agriculture and Life Science*, *54*(4), 69–74. https://doi.org/10.14397/jals.2020.54.4.69

Peluso, I. (2019). Dietary antioxidants: Micronutrients and antinutrients in physiology and pathology. *Antioxidants*, *8*(12), 642. https://doi.org/10.3390/ANTIOX8120642

Ramin, M., Yaakub, H., Alimon, A. R., & Jelan, Z. A. (2011). Effects of fungal treatment on the *in vitro* degradation of cassava. *Livestock Research for Rural Development*, *23*(7), 1–6.

Rodríguez-Martínez, B., Gullón, B., & Yáñez, R. (2021). Identification and recovery of valuable bioactive compounds from potato peels: A comprehensive review. *Antioxidants*, *10*(10), 1630. https://doi.org/10.3390/antiox10101630

Ru, W., Pang, Y., Gan, Y., Liu, Q., & Bao, J. (2019). Phenolic compounds and antioxidant activities of potato cultivars with white, yellow, red and purple flesh. *Antioxidants*, *8*(10), 419. https://doi.org/10.3390/antiox8100419

SAS. (2011). *SAS/STAT 9.3 User's guide*. SAS Institute Inc.

Sepelev, I., & Galoburda, R. (2015). Industrial potato peel waste application in food production: A review. *Research for Rural Development*, *1*, 130–136.

Sulaiman, C. T., & Balachandran, I. (2012). Total phenolics and total flavonoids in selected Indian medicinal plants. *Indian Journal of Pharmaceutical Sciences*, *74*(3), 258–260. https://doi.org/10.4103/0250-474x.106069

Taie, H. A. A., Abd-Alla, H. I., Ali, S. A., & Aly, H. F. (2015). Chemical composition and biological activities of two *Solanum tuberosum* cultivars grown in Egypt. *International Journal of Pharmacy and Pharmaceutical Sciences*, *7*(6), 311–320.

Valcarcel, J., Reilly, K., Gaffney, M., & O'Brien, N. (2015). Total carotenoids and L-ascorbic acid content in 60 varieties of potato (*Solanum tuberosum* L.) grown in Ireland. *European Potato Journal*, *58*, 29–41. https://doi.org/10.1007/s11540-014-9270-4

Valiñas, M. A., Lanteri, M. L., Have, A. T., & Andreu, A. B. (2017). Chlorogenic acid, anthocyanin and flavan-3-ol biosynthesis in flesh and skin of Andean potato tubers (S*olanum tuberosum* subsp. *andigena*). *Food Chemistry*, *229*, 837–846. https://doi.org/10.1016/j.foodchem.2017.02.150

Wu, J., Wang, L., Du, X., Sun, Q., Wang, Y., Li, M., Zang, W., Liu, K., & Zhao, G. (2018). α-solanine enhances the chemosensitivity of esophageal cancer cells by inducing microRNA-138 expression. *Oncology Reports*, *39*(3), 1163–1172. https://doi.org/10.3892/OR.2018.6187

Yılmaz, A., Yıldız, S., Kılıç, C., & Can, Z. (2017). Total phenolics, flavonoids, tannin contents and antioxidant properties of *Pleurotus ostreatus* cultivated on different wastes and sawdust. *International Journal of Secondary Metabolite*, *4*(1), 1–9. https://doi.org/10.21448/ijsm.252052

Zhao, D., & Shah, N. P. (2014). Changes in antioxidant capacity, isoflavone profile, phenolic and vitamin contents in soymilk during extended fermentation. *LWT - Food Science and Technology*, *58*(2), 454–462. https://doi.org/10.1016/j.lwt.2014.03.029

Zong, C., Xiao, Y., Shao, T., Chiou, J. A., Wu, A., Huang, Z., Chen, C., Jiang, W., Zhu, J., Dong, Z., Liu, Q., & Li, M. (2023). Alfalfa as a vegetable source of β-carotene: The change mechanism of β-carotene during fermentation, *Food Research International*, *172*, 113104. https://doi.org/10.1016/j.foodres.2023.113104

Pertanika J. Trop. Agric. Sci. 47 (4): 1343 - 1359 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Identification of Microorganisms Associated with Sea Cucumbers in Johor Coastal Seawater

**Siti Najihah Solehin[1], Kamarul Rahim Kamarudin[1]*, Nur Sabrina Badrulhisham[2] and 'Aisyah Mohamed Rehan[3]**

*[1]Department of Technology and Natural Resources, Faculty of Applied Sciences and Technology, Universiti Tun Hussein Onn Malaysia, 84600 Muar, Johor, Malaysia*
*[2]Department of Biotechnology and Breeding, Sime Darby Plantation Sdn Bhd, UPM-MTDC Technology Centre III, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
*[3]Department of Chemical Engineering Technology, Faculty of Engineering Technology, Universiti Tun Hussein Onn Malaysia, 84600 Muar, Johor, Malaysia*

## ABSTRACT

Sea cucumbers have been reported to host diverse microorganisms, including pigment-producing microorganisms. Investigating these microorganisms is essential for understanding ecological functions, potential biotechnology applications, and impacts on human health. However, despite their importance, the microbial diversity of sea cucumbers remains largely understudied. Thus, this study aims to identify the microorganisms associated with three species of sea cucumbers: *Holothuria pardalis*, *Holothuria leucospilota*, and *Holothuria scabra* collected from Johor coastal seawater. Identification of these isolates revealed that there were twenty-two strains of bacteria and three strains of fungi in total, representing 11 taxa, including 9 taxa from bacteria, namely *Staphylococcus*, *Bacillus*, *Brevibacillus*, *Psychrobacter*, *Stenotrophomonas*, *Chryseobacterium*, *Sphingomonas*, and *Pseudoxanthomonas*, and two taxa from fungi: *Aspergillus* and *Rhodotorula*. The isolates were identified using 16S rRNA for bacteria and internal transcribed spacer for fungi. Among these species, *Chryseobacterium* sp., *Sphingomonas* sp., and *Pseudoxanthomonas* sp. were first reported as part of the pigment-producing microorganisms found in sea cucumbers in Malaysia. Thus, these findings offer a novel insight into pigment-producing microorganisms in sea cucumbers and their potential as natural alternatives for colourants.

*Keywords*: 16S rRNA, bacteria, fungi, identification, ITS, phylogenetic analysis, pigment-producing microorganisms, sea cucumbers

ARTICLE INFO

*Article history:*
Received: 19 February 2024
Accepted: 26 March 2024
Published: 19 November 2024

DOI: https://doi.org/10.47836/pjtas.47.4.17

*E-mail addresses*:
snajihah91@gmail.com (Siti Najihah Solehin)
kamarulr@uthm.edu.my (Kamarul Rahim Kamarudin)
sabrina17797@gmail.com (Nur Sabrina Badrrulhisham)
aisyahr@uthm.edu.my ('Aisyah Mohamed Rehan)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

## INTRODUCTION

The sea cucumber, belonging to Holothuroidea and the phylum Echinodermata, is globally distributed in deep seas and benthic areas (Gianasi et al., 2021; Liu et al., 2023). Its consumption is widespread in China, Korea, Japan, Malaysia, Indonesia, and Russia due to its numerous biological activities (Hossain et al., 2020). It has leathery skin and a soft, cylindrical body with a single-branched gonad, which has been commercially utilised for food and health purposes over the past few decades (Halder & Pahari, 2020). These marine invertebrates, known for their application in food, cosmetics, and traditional medicine, encompass around 100 species harvested for commercial use, particularly in Asian countries like China, Indonesia, Japan, Korea, and Malaysia. Malaysia and Singapore were acknowledged as the Asia’s biggest importers of sea cucumbers (Louw & Bűrgener, 2020). More than 80 species of sea cucumbers have been documented in Malaysia (Solehin et al., 2021). The Aspidochirotida order exhibits Malaysia’s highest distribution and diversity of sea cucumbers.

The distribution of sea cucumbers in Malaysia has been documented across several Peninsular Malaysia, Sabah and Sarawak states. *Stichopus horrens* are the most popular species found in Langkawi and Pangkor Island, which are commercially exploited for medicine and food supplements (Kamarudin et al., 2015). In Sabah, four genera were recorded: *Holothuria*, *Stichopus*, *Actinopyga*, and *Molpadia*. Sea cucumbers are commercially marketed in Sabah and serve as food and traditional medicine. In addition, species of sea cucumbers from the Molpadiida order have been documented in Sarawak (Kamarudin et al., 2016). In Johor, *Holothuria* (*Halodeima*) *edulis* and *Stichopus chloronotus* were the first documentation of sea cucumbers in Pulau Aur, Johor, by Zulfigar et al. (2007).

For centuries, sea cucumbers have been popular throughout Asia as a medicine, delicacy and nutritious food (Song et al., 2020). Furthermore, today’s market offers a diverse array of products sourced from various parts of sea cucumbers, such as extracts from the body wall, liquid extracts, skin, and all body parts of sea cucumbers (Marchese et al., 2020; Tolon et al., 2021). Recent studies indicate that the isolation of culturable microorganisms associated with sea cucumbers has been identified with five genera: *Holothuria*, *Cucumaria*, *Stichopus*, *Apostichopus*, and *Eupentacta* (Wingfield et al., 2024).

Sea cucumbers obtain their food through the ingestion of marine sediments or filtering seawater (Mohsen et al., 2020). Besides, they also consume microfauna, bacteria, and decomposed organic and inorganic matter present on the surface of ocean sediments (Ennas et al., 2023). It is believed that seafloor bacterial colonies serve as both a direct food source and an indirect provider of essential nutrients for sea cucumbers (Chakraborty, 2022). Moreover, the natural products derived from bacteria associated with marine organisms offer a promising novelty for new research findings (Chu et al., 2020; Khalifa et al., 2019).

Therefore, this study aimed to identify microorganisms associated with three sea cucumbers: (1) *Holothuria* (*Lessonothuria*) *pardalis* Selenka (1867) from Pulau Tinggi, (2) *Holothuria* (*Metriatyla*) *scabra* (Jaeger, 1833) from Tanjong Surat, and (3) *Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt, 1835) collected from various locations in Johor's coastal seawater: Pulau Tinggi, Sedili Kechil, and Tanjung Surat using 16S rRNA and internal transcribed spacer (ITS). The discovery of these microorganisms, particularly those capable of producing pigments, could lead to new findings in the field of natural colourants.

## METHODOLOGY

### Research Sampling

Sea cucumbers were collected around coastal seawater during low tide from three different locations in Johor state, as shown in Figure 1, which were Pulau Tinggi (Figure 1a), Sedili Kechil (Figure 1b), and Tanjong Surat (Figure 1c). Three individuals, *Holothuria* (*Lessonothuria*) *pardalis* Selenka (1867) from Pulau Tinggi, *Holothuria* (*Metriatyla*) *scabra* (Jaeger, 1833) from Tanjong Surat, and *Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt, 1835) from Sedili Kechil, were sampled. The position and sampling sites were marked using the Global Positioning System (GPS) at 2.3047° N, 104.1176° E for Pulau Tinggi, 1.5876° N, 104.1466° E for Tanjong Surat, and 1.8258° N, 104.1587° E for Sedili Kechil. Fresh specimens of sea cucumbers were stored in ice boxes containing seawater during sampling for short-term storage. For long-term storage, the specimens were stored in a -20°C chest freezer with proper cataloguing (Kamarudin & Rehan, 2018).

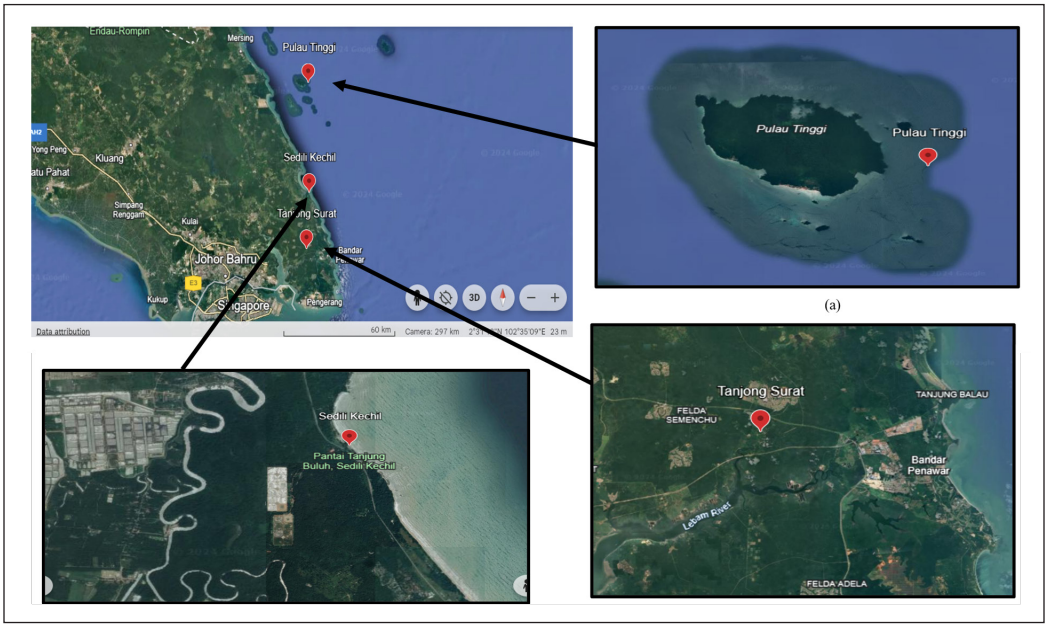



*Figure 1*. Sampling location for (a): Pulau Tinggi, (b): Sedili Kechil, and (c): Tanjong Surat (adapted from Google map images)

**Culture Media and Cultivation**

All specimens were dissected with a sterile blade in the Biological Safety Cabinet in the Food Microbiology Laboratory at the Faculty of Applied Science and Technology, Universiti Tun Hussein Onn Malaysia (UTHM), Pagoh, to mitigate possible contamination from the surroundings. All bacteria and fungi strains from the three sea cucumbers were isolated from their external body parts: the ventral podium, anus, and tentacles, as well as their internal body parts: coelomic fluid, the stomach, the cloaca, the respiratory tree, and the intestine. Each specimen body was swabbed using a sterile cotton swab and spread over Tryptone Glucose Yeast Extract (TGYE) agar (HiMedia Laboratories Private Limited, India) at pH 6.8 (Bajwa et al., 2018). The morphologies of the bacterial colonies were evaluated after overnight incubation at 37°C (since the average seawater temperature recorded in the sample sites was 37°C), and various colonies were repeatedly sub-cultured in new TGYE agar to purify each target individual of bacterium and fungus.

**DNA Extraction, Amplification, and Sequencing**

The FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., Taiwan) was used to extract total DNA from each bacterium and fungus associated with sea cucumbers. The bacterial strains' 16S rRNA genes were amplified using primers with V3-V4 target regions, which are the S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and the S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') primers, and the expected length of the amplified polymerase chain reaction (PCR) products is approximately 464 bp (Klindworth et al., 2013). As for fungal identification, two universal primers, ITS1 (forward) and ITS4 (reverse), were used for the isolation of the ITS region, which are ITS1 (5' – TCCGTAGGTGAACCTGCGG – 3'; 19 bases) and ITS4 (5'– TCCTCCGCTTATTCATATGC–3'; 20 bases) (Mirhendi et al., 2007), with an expected length of the amplified PCR product of approximately 800 bp. The PCR reaction mixture was prepared to a total volume of 25 µl comprising 12.5 µl PCR master mix (Axil Scientific Pte Ltd, Singapore), 1.5 µl of each primer (forward and reverse primer) (Axil Scientific Pte Ltd. Singapore), 6.5 µl of DNA sample and 3.0 µl of sterile ultra-pure water. Amplification was conducted using a PCR Max Alpha Cycler (Cole-Parmer, USA) with the following temperature profiles: for bacteria, initial denaturation for 3 min at 95°C was followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 5 min; for fungi, initial denaturation for 5 min at 95°C was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 1 min, and a final extension step at 72°C for 8 min.

Subsequently, sequences were visualised and edited using Chromas Version

2.6.6 (http://www.technelysium.co-m.au/chromas.html). The results were obtained as nucleotides in FASTA format. Species identification was performed using the resultant nucleotide base pairs through the Basic Local Alignment Search Tool (BLAST) algorithm analysis by direct blasting on http://blast.ncbi.nlm.nih.gov (Altschul et al., 1990). A read was performed for each set of isolates, and the top hit with a minimum E-score for every BLAST result showing the species name was utilised to name the specific organism. Similarity values of 99 to 100% indicated the same species. In comparison, 95 to 99% similarity values were regarded as the same genus, and values below 95% were categorised within the same family.

### Phylogenetic Analyses

The neighbour-joining (NJ) tree and the maximum likelihood (ML) tree were reconstructed using MEGA 11. A distance matrix was generated through pairwise alignment of the sequences, and the neighbour-joining method (Saitou & Nei, 1987) was employed to construct a phylogenetic tree based on this matrix. The branch lengths of the resulting tree were proportional to the estimated divergence along each branch. Confidence levels of the phylogenies were determined using the bootstrap method of Felsenstein (1985). The Kimura 2-parameter (K2P) nucleotide substitution model was applied for bacterial sequences, while the K2P with gamma distribution was employed for fungal sequences.

### GenBank Submission

The BankIt sequence submission tool takes precedence in organising the sequence data for GenBank submission. Subsequently, all sequences were submitted to GenBank: each acquires a distinct accession number serving as a unique identifier in the GenBank database. BankIt thoroughly examines submissions, detects common errors, and utilises Vecscreen, a variant of BLASTn, to identify any potential vector contamination (Benson et al., 2018).

## RESULTS

Twenty-five pure cultures were isolated from four body parts of the sampled sea cucumbers collected from three locations in Johor's coastal seawater: Pulau Tinggi, Tg. Surat, and Sedili Kechil. Genetic identification of all bacterial strains was conducted using 16S rRNA analysis, while fungal strains were identified using ITS. The bacterial strains exhibited an approximate length of 500 bp, as shown in Figure 2, whereas the fungal strains displayed an approximate length of 800 bp, as illustrated in Figure 3.

To ascertain the genus of the isolated bacteria and fungi, the 16S rRNA gene and ITS rRNA were sequenced. The resulting sequences were submitted to GenBank, NCBI. BLAST analysis of these DNA sequences identified strains belonging to ten different genera. Pairwise sequence comparisons were performed using BLAST analysis to determine sequence similarity (Kapli et al., 2020). The accession numbers for the isolated strains from GenBank were provided in Table 1.

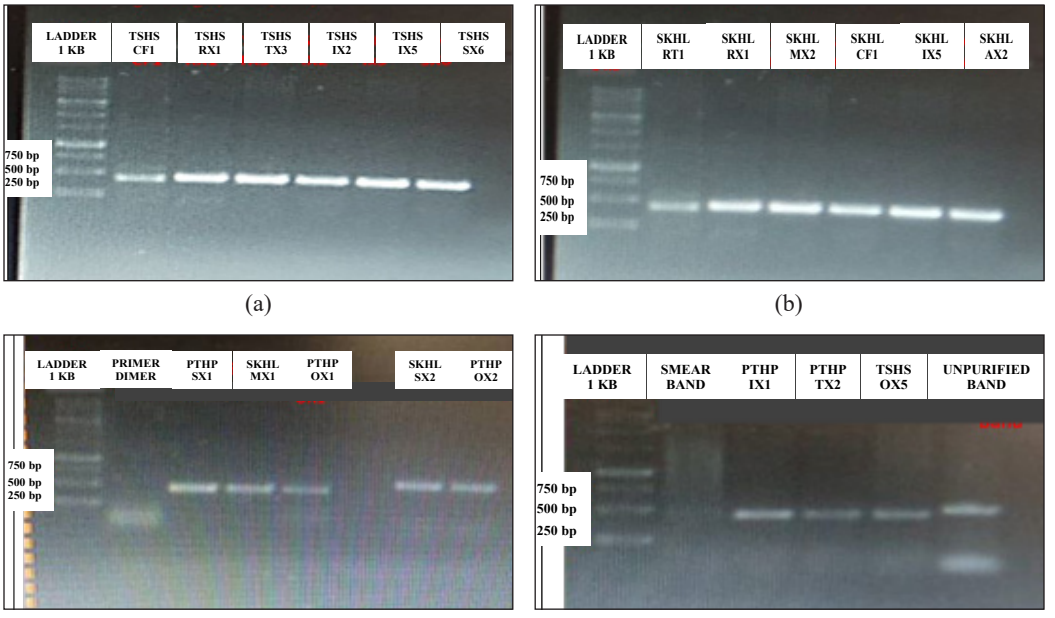



*Figure 2*. The DNA amplification for the isolated bacteria after polymerase chain reaction analysis on the agarose gel: (a) TSHS_CF1, TSHS_RX1, TSHS_TX3, TSHS_IX2, TSHS_IX5, and TSHS_SX6; (b) SKHL_RT1, SKHL_RX1, SKHL_MX2, SKHL_CF1, SKHL_IX5, and SKHL_AX2; (c) PTHP_SX1, SKHL_MX1, PTHP_OX1, SKHL_SX2, and PTHP_OX2; (d) PTHP_IX1, PTHP_TX2, and TSHS_OX5

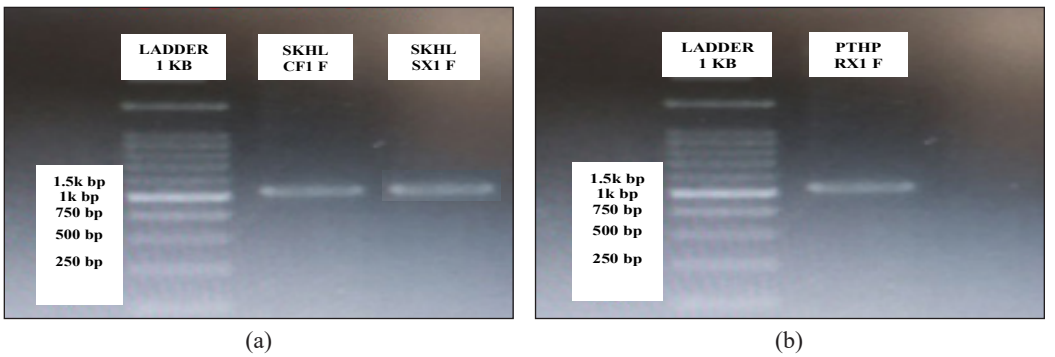



*Figure 3*. The DNA amplification for the isolated fungi after polymerase chain reaction analysis on the agarose gel: (a) SKHL_CF F and SKHL_SX1 F; (b) PTHP_RX1 F

Table 1
*Accession numbers of isolated bacterial and fungal strains deposited in GenBank*

| Isolates | GenBank accession number | Species identification | % similarities |
|---|---|---|---|
| PTHP_B12 | OP693446 | *Chryseobacterium* sp. | 100 |
| PTHP_AX1 | OP698237 | *Sphingomonas* sp. | 100 |
| PTHP_SX1 | OP684251 | *Staphylococcus* sp. | 100 |
| PTHP_TX2 | OP684252 | *Psychrobacter* sp. | 100 |

Table 1 *(continue)*

| Isolates | GenBank accession number | Species identification | % similarities |
|---|---|---|---|
| PTHP_IX1 | OP684253 | *Stenotrophomonas* sp. | 100 |
| PTHP_OX1 | OP684254 | *Brevibacillus* sp. | 99 |
| PTHP_OX2 | OP684255 | *Brevibacillus* sp. | 99 |
| TSHS_T1 | OP684296 | *Bacillus* sp. | 100 |
| TSHS_CF1 | OP684297 | *Brevibacillus* sp. | 99 |
| TSHS_IX2 | OP684298 | *Stenotrophomonas* sp. | 100 |
| TSHS_IX5 | OP684299 | *Pseudoxanthomonas* sp. | 99 |
| TSHS_SX6 | OP684300 | *Pseudoxanthomonas* sp. | 99 |
| TSHS_RX1 | OP698234 | *Pseudoxanthomonas* sp. | 99 |
| TSHS_TX1 | OP684301 | *Stenotrophomonas* sp. | 100 |
| TSHS_OX5 | OP698235 | *Pseudoxanthomonas* sp. | 99 |
| SKHL_CF1 | OP684290 | *Psychrobacter* sp. | 100 |
| SKHL_IX5 | OP684291 | *Stenotrophomonas* sp. | 100 |
| SKHL_AX2 | OP684246 | *Psychrobacter* sp. | 100 |
| SKHL_MX2 | OP684292 | *Psychrobacter* sp. | 100 |
| SKHL_RT1 | OP684293 | *Psychrobacter* sp. | 100 |
| SKHL_MX1 | OP684294 | *Psychrobacter* sp. | 100 |
| SKHL_SX2 | OP684295 | *Psychrobacter* sp. | 100 |
| SKHL_CF1 F | OP703319 | *Aspergillus* sp. | 100 |
| SKHL_SX1 F | OP703320 | *Aspergillus* sp. | 100 |
| PTHP_RX1 F | OP703321 | *Rhodotorula* sp. | 100 |

**NJ Tree**

The evolutionary history was elucidated using the NJ tree method (Saitou & Nei, 1987), with the optimal tree depicted in Figure 4. The percentage of replicate trees in which the linked taxa clustered together in the bootstrap test with 1,000 replicates was shown next to the branches (Felsenstein, 1985). Furthermore, evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004), quantified as the number of base substitutions per site. The study included 77 nucleotide sequences, with positions having less than 95% site coverage, and those with less than 5% alignment gaps, missing data, or ambiguous bases were excluded (utilising the partial deletion option). The resulting datasets comprised a total of 38 positions. Evolutionary patterns were analysed using MEGA11 (Tamura et al., 2021).

The bacterial phylogenetic tree depicted 77 taxa, comprising 74 ingroup taxa and three outgroup taxa. The outgroup taxa consisted of three individuals from the *Pseudomonas aeruginosa* species with GenBank accession no. NR026078, FJ9722533, and FJ972538, respectively. Within the tree, PTHP SX1 was found within the same clade as the *Staphylococcus* taxa, albeit with less than 50% bootstrap support. Similarly, TSHS_T1 clustered

alongside the *Bacillus* taxa, with less than 50% bootstrap support. Conversely, TSHS CF1, PTHP_OX2, and PTHP_OX2 formed a different cluster within the *Brevibacillus* taxa, supported by a 94% bootstrapping. Notably, the TSHS_CF1 isolates showed close proximity to *Brevibacillus parabrevis* NBRC 12334 (NR113589) with 94% bootstrap support. Moving on, SKHL_SX2, SKHL_RT1, SKHL_MX2, SKHL_MX1, SKHL_CF1, SKHL_AX2, and PTHP_TX2 were grouped under the *Psychrobacter* taxa. These isolates clustered alongside *Psychrobacter lutiphocae* IMMIIB L-1110 (NR044602) and *Psychrobacter sanguinis* 13983 (NR117833), supported by a 63% bootstrap. TSHS_IX2, SKHL_IX5, PTHP_IX1, and TSHS_TX1 were clustered under the *Stenotrophomonas* taxa. Specifically, TSHS_IX2 and SKHL_IX5 were grouped under the clade *Stenotrophomonas geniculate* ATCC 19374 (NR024708), while PTHP_IX1 clustered with the clade *Stenotrophomonas maltophilia* LMG958 (NR119220). Similarly, TSHS_TX1 was associated with *Stenotrophomonas malthophilia* IAM 12423 (NR041577). Additionally, PTHP_B12 isolates formed a cluster within the *Chryseobacterium* taxa, supported by a 55% bootstrap and grouped with the clade *Chryseobacterium timonianum* G972 (NR164881). Meanwhile, PTHP_AX1 was grouped under the *Sphingomonas* taxa with 95% bootstrap support and clustered alongside the clade *Sphingomonas olei* K-1-16 (NR157757). Lastly, TSHS_IX5, TSHS_OX5, TSHS_RX1, and TSHS_SX6 formed a cluster within the *Pseudoxanthomonas* taxa, supported by a 95% bootstrap and grouped with the clade *Pseudoxanthomonaskalamensis* JA40 (NR043110) as illustrated in Figure 4.

According to the fungal phylogenetic tree depicted in Figure 5, there are 19 taxa, comprising 18 ingroup taxa and one outgroup taxa, represented by *Candida albicans* CBS 562 (NG070791). SKHL_SX1 F and SKHL_CFI F were found to cluster together within the *Aspergillus* taxa, with a bootstrap support of 66%, alongside *Aspergillus arenarioides* CBS 138200 (NR135460). Conversely, PTHP_RX1 F isolates formed a cluster within the *Rhodotorula* taxa, with a strong bootstrap support of 99%, along with *Rhodotorula alborubescens* JCM5352 (NR153197).

**Maximum Likelihood Tree**

The phylogenetic tree, generated through maximum likelihood analysis, was constructed using the K2P model (Kimura, 1980) for bacteria and fungi. In the bacterial category, the tree with the highest log likelihood of -5235.88 was presented, with the percentage of trees wherein associated taxa clustered together indicated next to the branches. This analysis encompassed 77 nucleotide sequences, with a total of 1,455 positions in the final dataset.

According to Figure 6, PTHP_SX1 isolates formed a cluster within the *Staphylococcus* taxa, exhibiting strong bootstrap support at 99% — similarly, TSHS_T1 isolates clustered under the *Bacillus* taxa, with a strong 99% bootstrap support. PTHP_OX1, TSHS_CF1, and

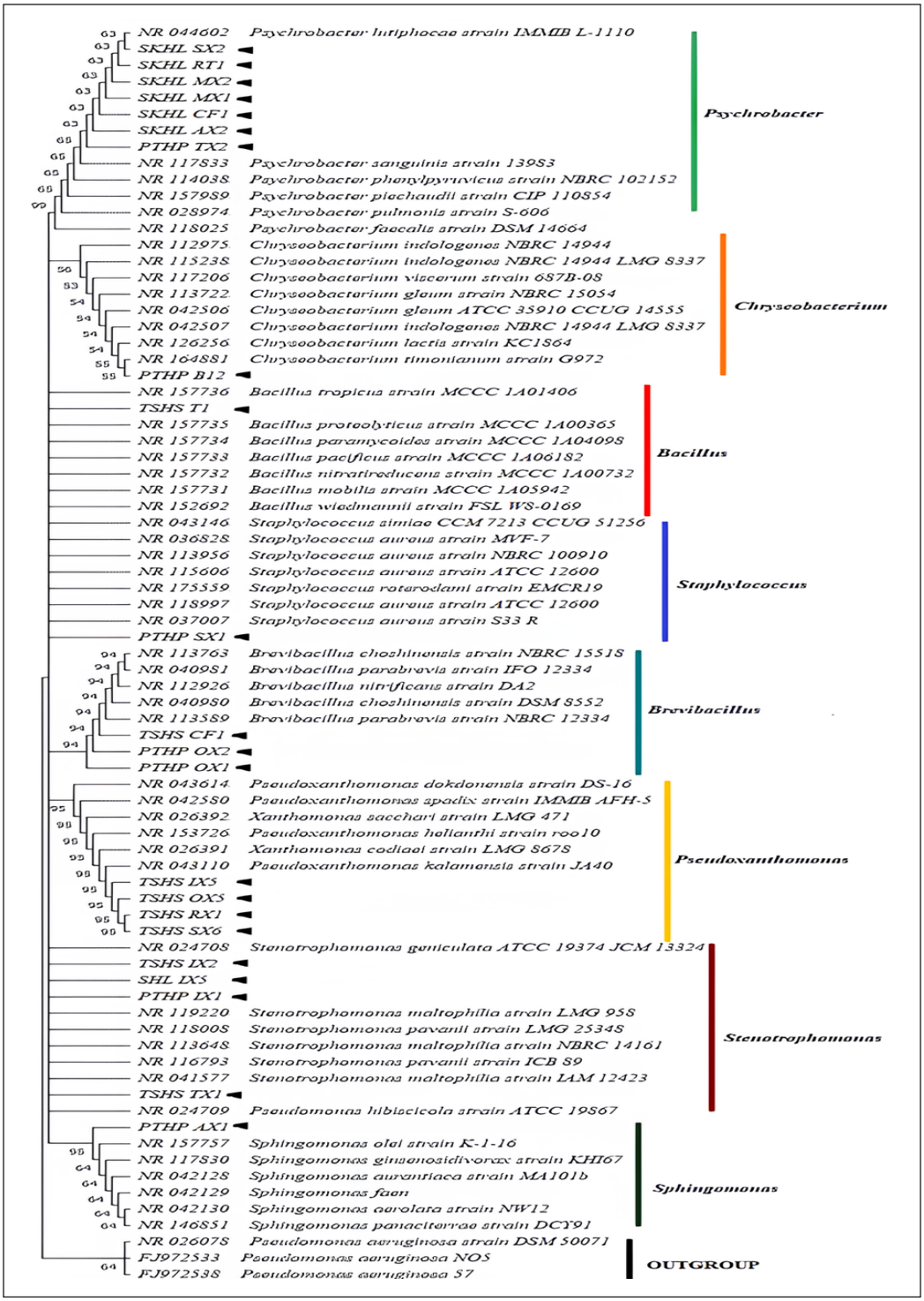



*Figure 4*. Neighbour-joining tree of 8 taxa of bacteria with *Peudomonas aeruginosa* as an outgroup. Alignment was done using MUSCLE W, and the tree was calculated using MEGA11 based on 1,000 bootstraps

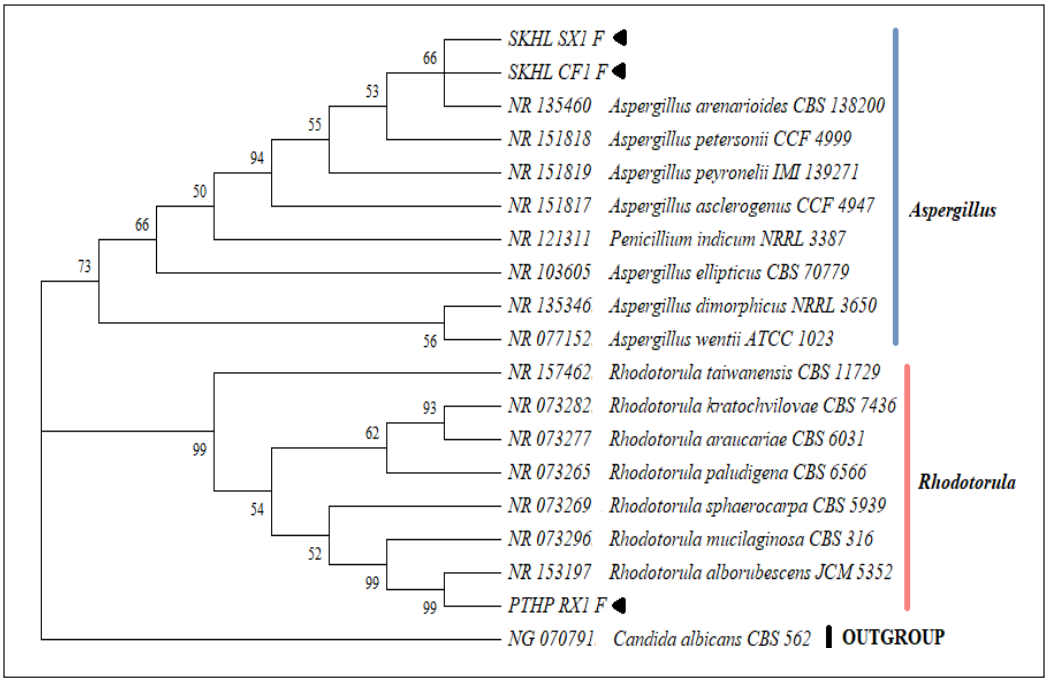



*Figure 5*. Neighbour-joining tree of 3 taxa of bacteria with *Candida albicans* as an outgroup. Alignment was done using MUSCLE W, and the tree was calculated using MEGA11 based on 1,000 bootstraps

PTHP_OX2 were grouped within the *Brevibacillus* taxa, supported by a 100% bootstrap. Additionally, SKHL_AX2, SKHL_CF1, SKHL_MX1, SKHL_RT1, SKHL_MX2, PTHP_TX2, and SKHL_SX2 formed a cluster under the *Psychrobacter* taxa, sharing a 99% bootstrap support and clustering with *Psychrobacter piechaudii* strain CIP 110854 (NR157989). SKHL_IX5, PTHP_IX1, and TSHS_IX2 clustered together with sequences from BLASTn, including *Pseudomonas hibiscicola* ATCC 19867 (NR024709), *Stenotrophomonas pavanii* LMG 25348 (NR041577), and *Stenotrophomonas malthophilia* IAM 12423 (NR041577), supported by a 59% bootstrap. PTHP_B12 exhibited 100% bootstrap support within the *Chryseobacterium* taxa, while PTHP_AX1 was classified under the *Sphingomonas* taxa, also with 100% bootstrap support. However, TSHS_TX1 and PTHP_AX1 formed a clade with a 45% bootstrap. Furthermore, TSHS_RX1, TSHS_SX6, TSHS_IX5, and TSHS_OX5 clustered within the *Pseudoxanthomonas* taxa, supported by a 70% bootstrap. Among them, TSHS_RX1, TSHS_SX6, and *Pseudoxanthomonas spadix* IMMIB AFH-5 (NR042580) formed a cluster, while TSHS_IX5 and TSHS_OX5 clustered with *Pseudoxanthomonas kalamensis* JA40 (NR043110), supported by a 54% bootstrap.

Similarly, the evolutionary history of the fungal tree was inferred using the K2P model with a discrete gamma distribution, accounting for evolutionary rate variations

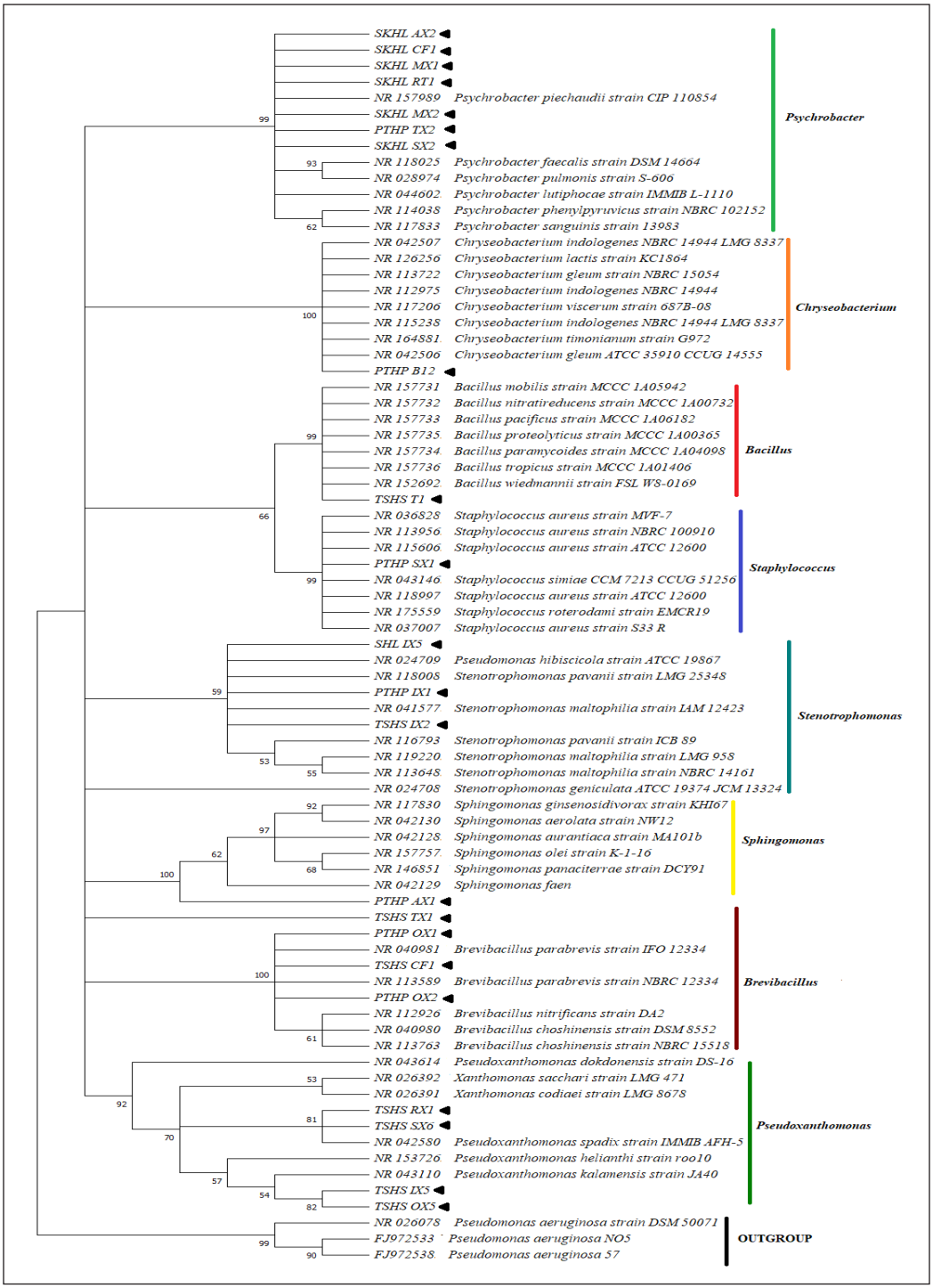



*Figure 6*. Maximum likelihood tree of 8 taxa of bacteria with *Pseudomonas aeruginosa* as an outgroup. Alignment was done using MUSCLE W, and the tree was calculated using MEGA11 based on 1,000 bootstraps

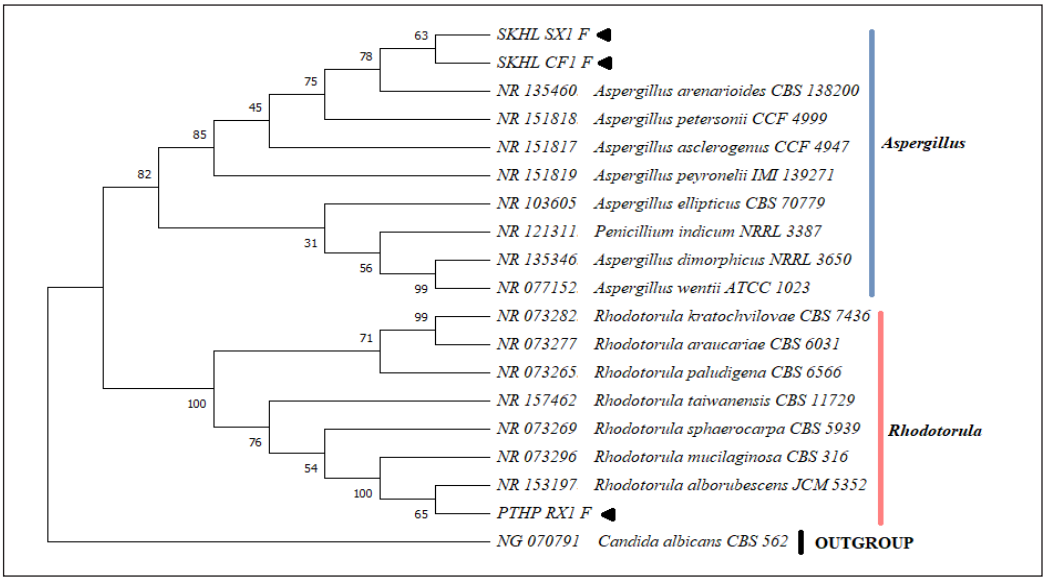



*Figure 7*. Maximum likelihood tree of 3 fungus taxa with *Candida albicans* as an outgroup. Alignment was done using MUSCLE W, and the tree was calculated using MEGA11 based on 1,000 bootstraps

among sites. This analysis involved 19 nucleotide sequences and incorporated 18 ingroup taxa and one outgroup taxa, represented by *Candida albicans* CBS 562 (NG070791), as illustrated in Figure 7. SKHL_SX1 F and SKHL_CF1 F formed a cluster within the *Aspergillus* taxa, supported by an 82% bootstrap. These isolates clustered alongside *Aspergillus arenarioides* CBS 138200 (NR135460) with a 78% bootstrap support. Conversely, PTHP_RX1 F was categorised under the *Rhodotorula* taxa with 100% bootstrap support and exhibited 65% bootstrap support with the *Rhodotorula alborubescens* JCM 5352 (NR152197) clade.

## DISCUSSION

The NJ and maximum likelihood analyses consistently revealed that isolates were grouped within the same taxa and confidently identified under their respective taxa: PTHP_SX1 as *Staphylococcus* sp.; TSHS_T1 as *Bacillus* sp.; TSHS_CF1, PTHP_OX2, and PTHP_OX1 as *Brevibacillus* sp.; SKHL_SX2, SKHL_RT1, SKHL_MX2, SKHL_MX1, SKHL_CF1, SKHL_AX2, and PTHP_TX2 as *Psychrobacter* sp.; TSHS_IX2, PTHP_IX1, and SKHL_IX5 as *Stenotrophomonas* sp.; PTHP_B12 as *Chryseobacterium* sp.; PTHP_AX1 as *Sphingomonas* sp.; TSHS_SX6, TSHS_RX1, TSHS_IX5, and TSHS_OX5 as *Pseudoxanthomonas* sp. However, while TSHS_TX1 was initially grouped under the *Stenotrophomonas* taxa in the NJ analysis, it exhibited weak bootstrap support and clustered with *Sphingomonas* sp. in the maximum likelihood analyses. The NJ method is the reconstruction for the distance-based method, while maximum likelihood is based on character-based methods (Kapli et

al., 2020). Most of microbial associated with selected sea cucumbers were predominantly normal microbiota.

*Psychrobacter* sp. was initially identified in the intestine of sea cucumber *Stichopus japonicus* (Gao et al., 2014) and later found in the external part of *H. leucospilota* collected from Lampung, Indonesia by Wibowo et al. (2019). However, there is no documentation of *Psychrobacter* sp. isolated from *H. leucospilota* and *H. pardalis* in Malaysia up to recent data. *Bacillus* sp., *Stenotrophomonas* sp., and *Brevibacillus* sp. were first reported by Lukman et al. (2014), who isolated them from the gastrointestine of *S. horrens* collected from Pangkor Island, Perak. Li et al. (2016) also reported *Bacillus* and *Brevibacillus* as the most abundant bacterial species in the gastrointestine. Kamarudin and Rehan (2018) also found *Bacillus* and *Brevibacillus* in *H. leucospilota* and *S. horrens* collected from Pangkor Island in 2018. *Staphylococcus* sp., isolated from the coelomic fluid of *H. leucospilota*, was first documented by Kamarudin et al. (2013) in Pangkor Island, Perak. However, no records of *Chryseobacterium* sp., *Pseudoxanthomonas* sp., and *Sphingomonas* sp. isolated from sea cucumber species have been documented in Malaysia or other Asian countries. Although *Aspergillus* sp. is commonly found in *H. polii* (Marchese et al., 2020), there is no data on *Aspergillus* sp. in *H. pardalis*, especially in Malaysia. While *Rhodotorula* sp. was found in *Apostichopus japonicas* by Wang et al. (2015) and Yang et al. (2019), there is no recorded data for *H. pardalis* and other sea cucumbers collected in Malaysia. Intriguingly, no data on microorganisms isolated from sea cucumbers collected from Johor Island, Malaysia, has been documented.

Of the eleven different bacterial strains taxa identified, *Chryseobacterium* sp., *Sphingomonas* sp., and *Pseudoxanthomonas* sp. emerged as the most promising candidates for further evaluation as potential food-grade microbial pigments. *Chryseobacterium* species, belonging to the Flavobacteriaceae family, typically produce colonies ranging from pale yellow to orange due to carotenoid content or flexirubin-type pigments (Hugo et al., 2019), while *Sphingomonas* sp. is known to produce various pigments ranging from yellow, red, or orange to white or non-pigmented (Busse et al., 2003). In this analysis, *Sphingomonas* sp. exhibited a bright yellow pigment on TGYE agar. According to Lipski and Stackebrandt (2015), *Pseudoxanthomona*s sp. produces yellow pigment on agar. Hence, these three pigmented bacteria provided new insights and discoveries on natural colourants derived from microorganisms.

## CONCLUSION

In this study, a total of 22 bacterial strains and three fungal strains were successfully identified. The bacteria were categorised into seven clades: *Psychrobacter*, *Chryseobacterium*, *Bacillus*, *Brevibacillus*, *Staphylococcus*, *Pseudoxanthomonas*, *Stenotrophomonas*, and *Sphingomonas*. Meanwhile, the three fungal strains were classified under the *Aspergillus* and *Rhodotorula* clades. Notably, this study

marks the first documentation of these species in Pulau Tinggi, Tanjong Surat, and Sedili Kechil in Johor. Among these species, *Chryseobacterium* sp., *Sphingomonas* sp., and *Pseudoxanthomonas* were first documented as part of pigment-producing microorganisms found in sea cucumbers in Malaysia.

The microorganisms isolated in this study are interesting discoveries for future research applications. The pigment-producing microorganisms can be further evaluated in terms of their potential and performance, such as growth factor, which can be used in colourant industries as one of the natural colourant alternatives.

## ACKNOWLEDGEMENTS

Communication of this research is made possible through monetary assistance from Universiti Tun Hussein Onn Malaysia (UTHM) and the UTHM Publisher's Office via Publication Fund E15216. This research was also supported by the Ministry of Higher Education Malaysia through the Fundamental Research Grant Scheme (FRGS) cycle 01/2019 (Grant ID FRGS/1/2019/WAB09/UTHM/03/2) and Postgraduate Research Grant (Grant ID H416, UTHM).

## REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Bajwa, K., Bishnoi, N. R., Toor, M., Gupta, S., Sharma, P., Kirrolia, A., Kumar, S. S., Sharma, J., & Selvan, S. T. (2018). Isolation, screening, characterization of indigenous oleaginous bacteria: Evaluation of various carbon and nitrogen sources as substrates for single celled oil producing bacteria. *Asian Journal of Biotechnology and Bioresource Technology*, *3*(1), 1–12. https://doi.org/10.9734/AJB2T/2018/39260

Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Ostell, J., Pruitt, K. D., & Sayers, E. W. (2018). GenBank. *Nucleic Acids Research*, *46*(D1), D41–D47. https://doi.org/10.1093/nar/gkx1094

Brandt, J. F. (1835). *Prodromus descriptionis animalium Ab H. Mertensio in orbis terrarum circumnavigatione observatorum* [Prodrome of the description of animals observed by H. Mertensio in the circumnavigation of the world]. Harvard University Press.

Busse, H.-J., Denner, E. B. M., Buczolits, S., Salkinoja-Salonen, M., Bennasar, A., & Kämpfer, P. (2003). *Sphingomonas aurantiaca* sp. nov., *Sphingomonas aerolata* sp. nov. and *Sphingomonas faeni* sp. nov., air- and dustborne and Antarctic, orange-pigmented, psychrotolerant bacteria, and emended description of the genus *Sphingomonas*. *International Journal of Systematic and Evolutionary Microbiology*, *53*(5), 1253–1260. https://doi.org/10.1099/ijs.0.02461-0

Chakraborty, S. K. (2022). Ocean ecosystem and its multidimensional eco-functionality and significance. In R. Brinkmann (Ed.), *The Palgrave handbook of global sustainability* (pp. 1–45). Palgrave Macmillan. https://doi.org/10.1007/978-3-030-38948-2_37-1

Chu, L., Huang, J., Muhammad, M., Deng, Z., & Gao, J. (2020). Genome mining as a biotechnological tool for the discovery of novel marine natural products. *Critical Reviews in Biotechnology*, *40*(5), 571–589. https://doi.org/10.1080/07388551.2020.1751056

Ennas, C., Pasquini, V., Abyaba, H., Addis, P., Sarà, G., & Pusceddu, A. (2023). Sea cucumbers bioturbation potential outcomes on marine benthic trophic status under different temperature regimes. *Scientific Reports*, *13*, 11558. https://doi.org/10.1038/s41598-023-38543-6

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, *39*(4), 783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

Gao, F., Tan, J., Sun, H., & Yan, J. (2014). Bacterial diversity of gut content in sea cucumber (*Apostichopus japonicus*) and its habitat surface sediment. *Journal of Ocean University of China*, *13*, 303–310. https://doi.org/10.1007/s11802-014-2078-7

Gianasi, B. L., Hamel, J.-F., Montgomery, E. M., Sun, J., & Mercier, A. (2021). Current knowledge on the biology, ecology, and commercial exploitation of the sea cucumber *Cucumaria frondosa*. *Reviews in Fisheries Science and Aquaculture*, *29*(4), 582–653. https://doi.org/10.1080/23308249.2020.1839015

Halder, D., & Pahari, S. K. (2020). An overviw of sea cucumber: chemistry and pharmacology of its metabolites. *Indian Research Journal of Pharmacy and Science*, *7*(2), 2277–2298. https://doi.org/10.21276/irjps.2020.7.2.19

Hossain, A., Dave, D., & Shahidi, F. (2020). Northern sea cucumber (*Cucumaria frondosa*): A potential candidate for functional food, nutraceutical, and pharmaceutical sector. *Marine Drugs*, *18*(5), 274. https://doi.org/10.3390/md18050274

Hugo, C., Bernardet, J.-F., Nicholson, A., & Kämpfer, P. (2019). Chryseobacterium. Wiley. https://doi.org/10.1002/9781118960608.gbm00301.pub2

Jaeger, G. F. (1833). *De Holothuriis* [Of the *Holothuria*]. https://www.biodiversitylibrary.org/page/10588969#page/5/mode/1up

Kamarudin, K. R., & Rehan, M. M. (2018). Gram-positive bacteria with commercial potential from the gastrointestines of *Holothuria* (*Mertensiothuria*) *Leucospilota* (*Timun Laut*) and *Stichopus Horrens* (*Gamat*) from Malaysian waters. *Pertanika Journal of Tropical Agricultural Science*, *41*(2), 605–620.

Kamarudin, K. R., Ngah, N., Hamid, T. H. T. A., & Susanti, D. (2013). Isolation of a pigment-producing strain of *Staphylococcus kloosii* from the respiratory tree of *Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt 1835) from Malaysian waters. *Tropical Life Sciences Research*, *24*(1), 85–100.

Kamarudin, K. R., Rehan, M. M., Noor, H. M., Ramly, N. Z., & Rehan, A. M. (2016). 16S rRNA barcoding technique for species identification of processed sea cucumbers from selected Malaysian markets. *Journal of Science and Mathematics Letters*, *4*, 10–23.

Kamarudin, K. R., Usup, G., Hashim, R., & Rehan, M. M. (2015). Sea cucumber (Echinodermata: Holothuroidea) species richness at selected localities in Malaysia. *Pertanika Journal of Tropical Agricultural Science*, *38*(1), 7–32.

Kapli, P., Yang, Z., & Telford, M. J. (2020). Phylogenetic tree building in the genomic age. *Nature Reviews Genetics*, *21*, 428–444. https://doi.org/10.1038/s41576-020-0233-0

Khalifa, S. A. M., Elias, N., Farag, M. A., Chen, L., Saeed, A., Hegazy, M.-E. F., Moustafa, M. S., Abd El-Wahed, A., Al-Mousawi, S. M., Musharraf, S. G., Chang, F.-R., Iwasaki, A., Suenaga, K., Alajlani, M., Göransson, U., & El-Seedi, H. R. (2019). Marine natural products: A source of novel anticancer drugs. *Marine Drugs*, *17*(9), 491. https://doi.org/10.3390/md17090491

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal

RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*(1), e1. https://doi.org/10.1093/nar/gks808

Li, F., Gao, F., Tan, J., Fan, C., Sun, H., Yan, J., Chen, S., & Wang, X. (2016). Characterization and identification of enzyme-producing microflora isolated from the gut of sea cucumber *Apostichopus japonicus*. *Chinese Journal of Oceanology and Limnology*, *34*, 153–162. https://doi.org/10.1007/s00343-015-4149-z

Lipski, A., & Stackebrandt, E. S. (2015). Pseudoxanthomonas. Wiley. https://doi.org/10.1002/9781118960608.gbm01234

Liu, H., Xue, C., & Li, Z. (2023). Diversity, distribution, and biology of sea cucumber. In C. Xue (Ed.), *Advances in sea cucumber processing technology and product development* (pp. 1–20). Springer. https://doi.org/10.1007/978-3-031-16512-2_1

Louw, S., & Bűrgener, M. (2020). *A rapid assessment of the sea cucumber trade from Africa to Asia*. TRAFFIC International.

Lukman, A. L., Nordin, N. F. H., & Kamarudin, K. R. (2014). Microbial population in the coelomic fluid of *Stichopus chloronotus* and *Holothuria* (*Mertensiothuria*) *leucospilota* collected from Malaysian waters. *Sains Malaysiana*, *43*(7), 1013-1021.

Marchese, P., Garzoli, L., Gnavi, G., O'Connell, E., Bouraoui, A., Mehiri, M., Murphy, J. M., & Varese, G. C. (2020). Diversity and bioactivity of fungi associated with the marine sea cucumber *Holothuria poli*: Disclosing the strains potential for biomedical applications. *Journal of Applied Microbiology*, *129*(3), 612–625. https://doi.org/10.1111/jam.14659

Mirhendi, H., Diba, K., Rezaei, A., Jalalizand, N., Hosseinpur, L., & Khodadadi, H. (2007). Colony PCR is a rapid and sensitive method for DNA amplification in yeasts. *Iran Journal of Public Health*, *36*(1), 40–44.

Mohsen, M., Zhang, L., Sun, L., Lin, C., Liu, S., Wang, Q., & Yang, H. (2020). A deposit-feeder sea cucumber also ingests suspended particles through the mouth. *Journal of Experimental Biology*, *223*(24), jeb230508. https://doi.org/10.1242/jeb.230508

Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Selenka, E. (1867). *Beiträge zur anatomie und systematik der* Holothurien [Contributions to the anatomy and systematics of *Holothuria*] (Vol. 17). *W. Engelmann* Publisher.

Solehin, S. N., Kamarudin, K. R., Akashah, N., Rehan, A. M., Bakar, M. A. L. A., Badrulhisham, N. S., Rahman, N. S. A., Akma, U. N., Shahdan, F., Azman, H., Fadzil, S. N. M., Faid, N. H. M., Zaman, N. S. S., Legiman, M. I., Salleh, F. M., & Esa, Y. (2021). Species identification and relationship of sea cucumber species from Pulau Tinggi and Sedili Kechil, Johor based on ossicle shape. *Journal of Sustainable Natural Resources*, *2*(1), 38-45. https://doi.org/10.30880/jsunr.2021.02.01.006

Song, Z., Li, H., Wen, J., Zeng, Y., Ye, X., Zhao, W., Xu, T., Xu, N., & Zhang, D. (2020). Consumers' attention on identification, nutritional compounds, and safety in heavy metals of Canadian sea cucumber in Chinese food market. *Food Science and Nutrition*, *8*(11), 5962–5975. https://doi.org/10.1002/fsn3.1882

Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences*, *101*(30), 11030–11035. https://doi.org/10.1073/pnas.0404206101

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Molecular Biology and Evolution*, *38*(7), 3022–3027. https://doi.org/10.1093/molbev/msab120

Tolon, M. T., Karacalar, U., & Şirin, C. (2021). Observation of *Vibrio mediterranei* (Pujalte and Garay 1986) / *Vibrio shiloi* (Kushmaro et al. 2001) bacteria from skin ulcers of the cultured sea cucumber *Holothuria poli* (Delle Chiaje, 1823). *Ege Journal of Fisheries and Aquatic Sciences*, *38*(3), 393–397. https://doi.org/10.12714/egejfas.38.3.16

Wang, J.-H., Zhao, L.-Q., Liu, J.-F., Wang, H., & Xiao, S. (2015). Effect of potential probiotic *Rhodotorula benthica* D30 on the growth performance, digestive enzyme activity and immunity in juvenile sea cucumber *Apostichopus japonicus*. *Fish and Shellfish Immunology*, *43*(2), 330–336. https://doi.org/10.1016/j.fsi.2014.12.028

Wibowo, J. T., Kellermann, M. Y., Versluis, D., Putra, M. Y., Murniasih, T., Mohr, K. I., Wink, J., Engelmann, M., Praditya, D. F., Steinmann, E., & Schupp, P. J. (2019). Biotechnological potential of bacteria isolated from the sea cucumber *Holothuria leucospilota* and *Stichopus vastus* from Lampung, Indonesia. *Marine Drugs*, *17*(11), 635. https://doi.org/10.3390/md17110635

Wingfield, L. K., Atcharawiriyakul, J., & Jitprasitporn, N. (2024). Diversity and characterization of culturable fungi associated with the marine sea cucumber *Holothuria scabra*. *PLOS One*, *19*(1), e0296499. https://doi.org/10.1371/journal.pone.0296499

Yang, G., Tian, X., & Dong, S. (2019). *Bacillus cereus* and rhubarb regulate the intestinal microbiota of sea cucumber (*Apostichopus japonicus* Selenka): Species-species interaction, network, and stability. *Aquaculture*, *512*, 734284. https://doi.org/10.1016/j.aquaculture.2019.734284

Zulfigar, Y., Sim, Y. K., & Aileen Tan, S. H. (2007). The distribution of sea cucumbers in Pulau Aur, Johore, Malaysia. *Publications of the Seto Marine Biological Laboratory*, *8*, 73–86. https://doi.org/10.5134/70908

Pertanika J. Trop. Agri. Sci. 47 (4): 1361 - 1378 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Effects of Beneficial Bacterial Inoculation on Arsenic Hyperaccumulation Ability of *Pteris vittata* under Planthouse Conditions

**Aminu Salisu Mu'azu[1,2], Hazzeman Haris[1], Kamarul Zaman Zarkasi[1], Nyok-Sean Lau[3] and Amir Hamzah Ahmad Ghazali[1]***

*[1]School of Biological Sciences, Universiti Sains Malaysia, 11800 Pulau Pinang, Malaysia*
*[2]Department of Science Laboratory Technology, Hussaini Adamu Federal Polytechnic P.M.B. 5004, Kazaure, Jigawa State, Nigeria*
*[3]Center for Chemical Biology, Universiti Sains Malaysia, Blok B No. 10, Persiaran Bukit Jambul, 11900 Bayan Lepas, Pulau Pinang, Malaysia*

## ABSTRACT

The widespread problem of arsenic buildup in soil is often addressed through phytoremediation techniques. *Pteris vittata*, an herbaceous fern known as the Chinese ladder brake, exhibits exceptional arsenic hyperaccumulation, storing over 27,000 mg of arsenic per kilogram in its aboveground biomass as dry weight. Planting *P. vittata* in arsenic-contaminated areas emerges as a promising strategy, facilitating the fern's absorption and accumulation of arsenic from the soil and, consequently, mitigating environmental arsenic levels. This study was conducted to assess the impact of two bacterial strains, *Bacillus* sp. 3P20 (CCB-MBL 5013) and *Enterobacter* sp. 3U4 (CCB-MBL 5014), on arsenic hyperaccumulation by *P. vittata*. The total arsenic content in both soil and plant samples was quantified using inductively coupled plasma-optical emission spectrometry. The results showed a significant difference ($P < 0.0001$) between *P. vittata* inoculated with *Bacillus* sp. 3P20 and spiked with 200 and 500 mg/L arsenic, having a total arsenic content of 240 and 255.25 mg/kg, respectively, compared to the control (un-inoculated), which has 143 mg/kg. Additionally, there was a significant difference ($P < 0.0001$) between *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 200 and 500 mg/L arsenic. Although no significant increase in the leaf greenness value of the plant was observed in the first and fourth weeks a noteworthy

ARTICLE INFO

*Article history:*
Received: 26 January 2024
Accepted: 03 May 2024
Published: 29 November 2024

DOI: https://doi.org/10.47836/pjtas.47.4.18

E-mail addresses:
aminsalis26@yahoo.com (Aminu Salisu Mu'azu)
hazzeman@usm.my (Hazzeman Haris)
kamarul.zarkasi@usm.my (Kamarul Zaman Zarkasi)
nyoksean@usm.my (Nyok-Sean Lau)
amirhg@usm.my (Amir Hamzah Ahmad Ghazali)
*Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

increase was recorded after the eighth week of transplanting. These indicate that the bacterial strains promoted plant growth and significantly enhanced the efficiency of arsenic hyperaccumulation by the fern.

*Keywords*: Arsenic, beneficial bacteria, contamination, hyperaccumulation, *Pteris vittata*, soil

## INTRODUCTION

Arsenic pollution is a global threat to plants, animals, and human health. Existing solutions are costly and ineffective (Alka et al., 2020). The widespread occurrence of arsenic pollution across different regions worldwide has raised significant concern. The high costs, disruptive effects, and potential generation of harmful by-products associated with traditional remediation methods have led to a notable shift towards exploring eco-friendly and sustainable approaches to address the problem of arsenic contamination (Manzoor et al., 2019). Bioremediation, using natural agents like microorganisms and plants, offers a promising, affordable, and eco-friendly alternative to traditional methods for effectively cleaning up polluted areas (Alka et al., 2020).

Arsenic-contaminated soils can be remediated by phytoremediation technology, an economical and environmentally friendly soil remediation technique (Manzoor et al., 2019). The method is a unique, cost-efficient, ecologically beneficial, and environmentally friendly soil remediation process. It utilizes hyper-accumulator plants, e.g., *P. vittata*, to remove metal pollutants from the soil.

Progress in arsenic removal from contaminated soils and water through phytoremediation has notably advanced with the identification of the hyperaccumulator *P. vittata*. *Pteris vittata*, commonly called Chinese brake fern, is an herbaceous plant native to China that can accumulate high arsenic in its fronds. It is the first identified arsenic-hyperaccumulator to store significant arsenic levels in its aboveground biomass. *Pteris vittata* has gained significant recognition for its exceptional ability to hyper-accumulate arsenic (Setyawan et al., 2021). It has the potential for phytoextraction of arsenic from polluted soils into harvestable parts (Popov et al., 2021). Its ability to store large quantities of arsenic in its plant tissue makes it a promising choice for phytoremediation in arsenic-contaminated soils (Setyawan et al., 2021).

Regardless of arsenic concentrations, species types, growth media, and treatment times, most arsenic accumulated in *P. vittata* is concentrated in the fronds, demonstrating efficient arsenic transfer from roots to fronds. The amounts of arsenic in the growth media and exposure duration correlate with the arsenic levels in the roots and fronds. The amount of arsenic in the fronds declined with the plant's aging. *Pteris vittata* can accumulate as much as 27,000 mg/kg of arsenic, with detrimental effects on plants becoming evident at approximately 10,000 mg/kg dry weight (J. Wang et al., 2002; Vandana et al., 2020). Arsenic is mainly kept in pinnae and rhizomes in the fronds of *P. vittata* (Tiwari et al., 2016). However,

it was absent in the cell walls, rhizoids, or reproductive regions. In contrast, non-accumulator plants typically experience adverse effects at 5-100 mg/kg dry weight levels of arsenic (Vandana et al., 2020). The concentration of arsenic was highest in the apical pinnae apex and gradually declined in the pinnae at lower places in the same frond. It was also more significant at the edge in single pinnae (Bui, 2017). Arsenic is taken up primarily by *P. vittata* and other plants as As(V), and they are more tolerant to it than As(III) (Yan et al., 2019; Yang et al., 2022).

Recent research indicates that the ability of *P. vittata* to hyper-accumulate arsenic is not solely a result of its inherent traits; instead, it is influenced significantly by the interactions with microorganisms in the rhizosphere. The rhizosphere is a dynamic environment where microorganisms flourish and engage in complex molecular communication with the plant. Soil bacteria are considered a preferred choice for phytoremediation research due to their ability to enhance host plants' growth and their resistance to heavy metals. Their ability to flourish in metal-polluted environments and support plant growth while eliminating heavy metals from the soil are significant factors in their high regard for phytoremediation studies (Tirry et al., 2018).

This study explores the relationship between locally isolated arsenic-tolerant bacteria *Bacillus* sp. 3P20 (CCB-MBL 5013) and *Enterobacter* sp. 3U4 (CCB-MBL 5014), and *P. vittata* in the processes of arsenic hyperaccumulation and plant growth promotion. The use of beneficial bacteria in phytoremediation shows promise as an eco-friendly and sustainable method to enhance arsenic removal from contaminated soils (Setyawan et al., 2021). A better understanding of the association between *P. vittata* and these microorganisms could lead to innovative and cost-effective strategies to tackle arsenic pollution, ultimately promoting a safe and healthier environment.

## MATERIALS AND METHODS

### Selection of Bacterial Isolates

One hundred and twenty-four (124) bacterial isolates were subjected to qualitative screening to assess their capacity for promoting plant growth through the production of plant growth-promoting (PGP) substances such as indole-3-acetic acid (IAA), siderophore, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and mineral solubilization, including phosphate, potassium, and silicate. The ten most promising strains demonstrating arsenic tolerance and PGP traits were selected for quantitative analysis. These PGP traits were then evaluated under arsenic stress across concentrations ranging from 50 to 2000 mg/L of As (III). *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 were selected for further investigation to determine their impact on the growth of *P. vittata* and their potential role in bioremediation efficiency (Muazu, 2024).

### Exposure of *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 to Arsenic Stress

The isolates were treated with 500 mg/L of sodium arsenite ($NaAsO_2$) and then observed under a scanning electron microscope (SEM, Leo Supra 50 VP, Germany) and transmission electron microscope (TEM, ZEISS Libra 120, Germany).

### SEM and TEM Sample Preparations

The sample for SEM was prepared using the hexamethyldisilazane (HMDS) method. A 24-hr bacterial culture was centrifuged at 1,000–2,000 x *g* for 15 min, and the pellet was resuspended in McDowell-Trump fixative prepared in 0.1 M phosphate buffer (pH 7.2, Sigma-Aldrich, USA), for overnight fixation. The resuspended sample was then centrifuged, and the supernatant was discarded. The pellet was then transferred to an Eppendorf tube and resuspended in 0.1 M phosphate buffer (Buffer wash 1). The process was repeated using the same buffer (Buffer wash 2). Then, the sample was centrifuged, and the pellet was resuspended in 1% osmium tetroxide (Polysciences, USA), prepared in the phosphate buffer above for at least 2 hr (post-fixation). The resuspended sample was then centrifuged, and the pellet was resuspended in distilled water (post-fix wash 1), and the process was repeated (post-fix wash 2). The sample was centrifuged, and the supernatant was discarded. The sample was dehydrated in various ethanol concentrations (Sigma-Aldrich, USA): 50% ethanol (10 min), 75% ethanol (10 min), 95% ethanol (10 min), and 100% ethanol (2 x 10 min). Dehydrated cells were immersed in 1–2 ml of HMDS (ALFA Chem, USA), for 10 min. The HMDS was decanted from the sample tube, and the vial with the cells was left in a desiccator to air-dry at room temperature. The dried cells were then mounted on an SEM specimen stub with double-sided sticky tape. The specimens were coated with gold and viewed in the SEM (Leo Supra 50 VP, Germany).

For TEM sample preparation, the procedure was the same as that for SEM up to the post-wash 2 step above. After that, the sample was centrifuged, and the supernatant was discarded. Then, the tube containing the fixed pellet cells was placed in a water bath at 45°C for 15–30 min. An agar solution of 3% (Oxoid, United Kingdom) was prepared by dissolving the agar in boiling distilled water. The solution was then poured into a still-molten tube and placed in the water bath at 45°C, maintaining the agar in liquid form. The warm agar was transferred to the tube containing the pellet using a warm pipette after the temperature of both the agar and the pellet had equilibrated to 45°C; the mixture was then stirred up to break the pellet into small blocks and suspended in the agar. The agar with the suspended pellet blocks was then immediately poured onto a clean glass slide and allowed to solidify within 1–2 min. The solidified agar containing the cells was cut into small cubes of 1 $mm^3$ with a sharp razor blade and then placed in a vial containing 50% ethanol (Sigma-Aldrich, USA). The cubes were then processed by dehydration in various ethanol concentrations: 50% ethanol (15 min), 75% ethanol (15 min), 95% ethanol (2 x 15 min), 100% ethanol (2 x 30 min),

and acetone (Bendosen, Malaysia) (2 x 10 min). Followed by infiltrating resin using the mixture of acetone: spurs resin (Sigma-Aldrich, USA) mix (1:1) in a rotator for 15–30 min. Then, it was infiltrated in spur's mix overnight, after which the resin was changed and allowed to stay in the rotator for 48 hr. Then, it was embedded and cured in an oven at 60°C for 12–48 hr. The cubes were then cut using an ultramicrotome, placed on a copper grid, stained, and viewed under TEM.

## Transplanting of *P. vittata* and Soil Sample Collection

Healthy *P. vittata*, which grows naturally in the field and are approximately of the same size (16 cm in height) and weight, were collected from the Universiti Sains Malaysia (USM) main campus and immediately transported to the plant house aseptically and then carefully rinsed with distilled water to remove dirt and soil remnants. The plants were selected, transplanted into polybags containing soils, and kept for one week of acclimatization. The plants were grown in a plant house with temperatures ranging from 22°C at night to 30°C during the day (Abou-Shanab et al., 2020; Q. Wang et al., 2011).

The soil sample was collected from the main campus of USM at latitude $05^0$ 21.341'N and longitude $10^0$ 018.048'E, at an elevation of 31 m. The soil was taken from the top layer (0–20 cm), where *P. vittata* grows naturally (Abou-Shanab et al., 2020). The soil was mixed in a sizable container, air-dried, crushed, and sieved with a 2-mm sieve. A total of 3 kg of soil was transferred into separate polybags, each measuring 18 cm in diameter and 13 cm in length (Abou-Shanab et al., 2020). The polybags were then grouped into three groups. The first group served as the control and contained 3 kg of untreated soil (Bui, 2017). The second group contained the same soil but was enriched with a 200 mg/L solution of sodium arsenite ($NaAsO_2$, Sigma-Aldrich, USA), while the last group was spiked with 500 mg/L of $NaAsO_2$ (Abou-Shanab et al., 2020). The bags were arranged in a randomized block design on the benches in the plant house for the experiment (Abou-Shanab et al., 2020; Bui, 2017).

## Inoculation of Bacterial Isolates

Twenty (20) ml of the 24-hr bacterial culture of *Bacillus* sp. 3P20 and *Enterobacter* 3U4 (OD600; 1.2) were inoculated into the corresponding plastic bag during the first and second week of transplanting. The setup was maintained and watered daily in the plant house for the eighth week, and the plant was uprooted after the eighth week of transplanting for arsenic content analysis. The arsenic content of *P. vittata* and the soil samples from the sampling site (natural mineral soils) was initially measured and recorded. Inductively coupled plasma optical emission spectroscopy (ICP-OES, Thermo-Fisher Scientific, USA), was employed to determine the concentrations of total and extractable metals in the soil samples (Abou-Shanab et al., 2020; Lampis et al., 2015; Q. Wang et al., 2011). The analysis was conducted by ALS Technichem (M) Sdn. Bhd. (Malaysia).

### Determination of the Leaf Chlorophyll Content of the Plant

The leaf chlorophyll content of the plants was evaluated at the first, fourth, and eighth week of transplanting non-destructively using a SPAD (Soil Plant Analysis Development) 502 chlorophyll meter (Konica Minolta, USA). The device uses light absorption to measure the leaf greenness of the plant, and the values were recorded accordingly (Nacoon et al., 2020). The *P. vittata* was harvested after the eighth week of transplanting. The roots of the plants were washed with clean running tap water to remove soils that adhered to the roots, and the plant samples were dried in an oven at 80°C for 3 days (Nacoon et al., 2020). Drying the samples in an oven at a high temperature helps to remove any moisture and preserve the samples for further analysis or storage (Q. Wang et al., 2011). The shoot and root dry weights were determined after harvesting the plants. The *P. vittata* samples were then ground using a mortar and pestle, and each was transferred into a Ziploc plastic bag and labeled accordingly. The samples were sent to the ALS Technichem (M) Sdn. Bhd. (Malaysia) to determine the total arsenic content.

### Detection of Plant and Soil Arsenic Content

The sample was prepared by digestion in an acidic solution with four replications each, and the digested sample was aspirated into ICP-OES. The intensity of characteristic light emitted by each excited element was observed to be proportional to its concentration. The arsenic decontamination was measured by evaluating arsenic levels in plant tissues after the experiment and comparing them with the initial and final soil arsenic concentrations to evaluate the arsenic uptake by *P. vittata* and the effects of the bacterial inoculation on the hyperaccumulation ability (Muazu, 2024).

### Assessment of the Efficiency of Phytoremediation

The efficiency of phytoremediation by the plant was determined by evaluating the bioconcentration factor (BCF). The following formula was used to calculate: BCF = C harvested tissue / C soil, where "C harvested tissue" denotes the metal concentration in various plant tissues, and "C soil" represents the metal concentration in soil measured in mg/kg (Debela et al., 2022).

### Data Analysis

The data obtained from this research were analyzed through analysis of variance (ANOVA) to determine if there were significant differences among treatments, using GraphPad Prism statistical software version 9.0 (Inc., USA). One-way and two-way ANOVA were used for the analysis, followed by Tukey's multiple comparison tests. The statistical differences were considered significant at $P < 0.05$.

## RESULTS

### SEM and TEM Analysis

The SEM images of the bacterial isolates grown without arsenic (control) and those

grown in 500 mg/L arsenic (III) revealed no significant changes in the treated cells of *Bacillus* sp. 3P20 compared to the untreated cells, indicating that the cells were unaffected by the exposure to 500 mg/L arsenic (Figure 1).

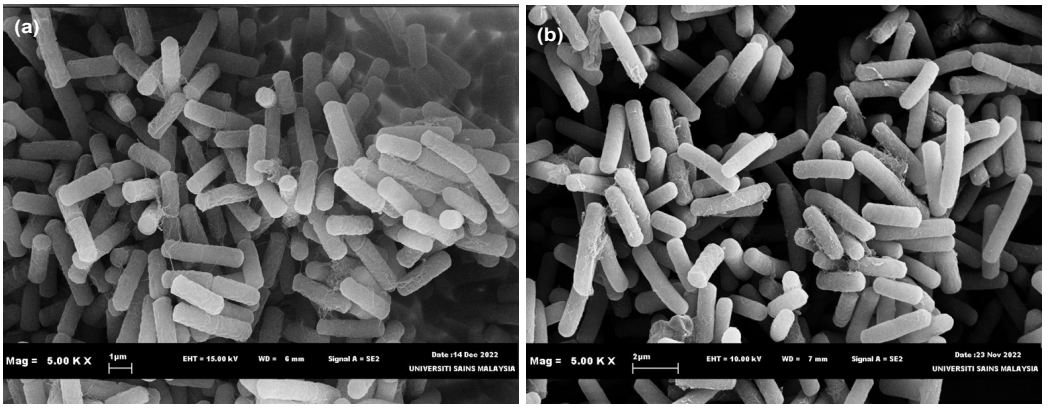



*Figure 1*. Scanning electron microscopy of *Bacillus* sp. 3P20. (a) Untreated (control) cells without the arsenic treatment; (b) Cells treated with 500 mg/L of arsenic

Similarly, there were no significant changes in the morphology of *Enterobacter* sp. 3U4 cells treated with 500 mg/L As(III) compared to the control (Figure 2).

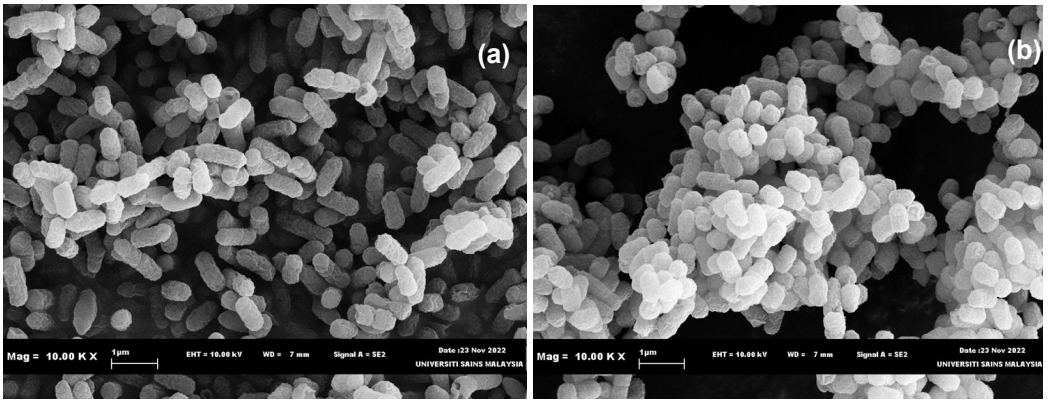



*Figure 2*. Scanning electron microscopy of *Enterobacter* sp. 3U4. (a) Untreated (control) cells without the arsenic treatment; (b) Cells treated with 500 mg/L arsenic

TEM was used to investigate the intracellular accumulation of arsenic. The TEM results for the treated and untreated (control) cells are presented in Figure 3 for *Bacillus* sp. 3P20 and Figure 4 for *Enterobacter* sp. 3U4. There were no observable differences between treated and untreated cells.

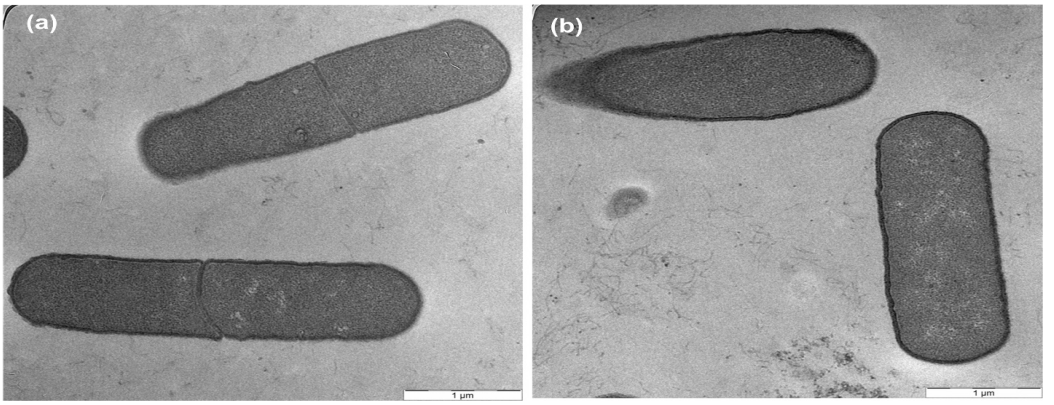



*Figure 3*. Transmission electron microscope of *Bacillus* sp.: (a) without arsenic exposure, the bacterial cells showed paired cells; (b) the bacterial cells exposed to 500 mg/L arsenic (sodium arsenite), indicating that the cells were unaffected by the treatment

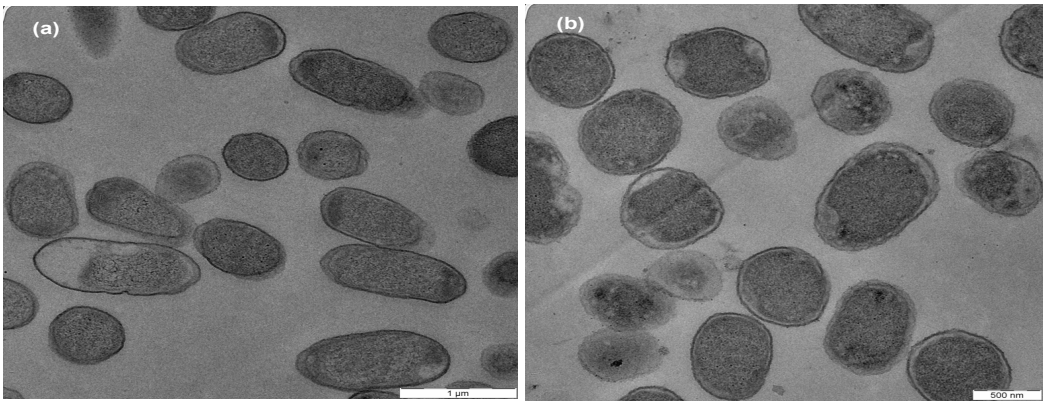



*Figure 4*. Transmission electron microscope of *Enterobacter* sp.: (a) the cells without arsenic exposure; (b) cells exposed to 500 mg/L of arsenic, indicating that the cells were unaffected by the arsenic treatment

## Total Arsenic Contents in Plant (*P. vittata*) and Soil Samples before Bacterial Inoculation

The total arsenic contents of plant and soil samples were determined and recorded prior to the commencement of the experiment as the initial arsenic concentration. The result shows that the total arsenic content of *P. vittata* in the samples ranged from 122 to 143 mg/kg, and that of the soil samples ranged between 5 to 6 mg/kg.

## Total Arsenic Contents in *P. vittata* after Bacterial Inoculation

From the result obtained, the total arsenic content in the frond of *P. vittata* after the experiment was observed to be higher in the plants inoculated with *Enterobacter* sp. 3U4 and spiked with 500 mg/L arsenic solution (499.5 mg), followed by the plant inoculated with 200 mg/L arsenic (239.5 mg/kg). There was a significant difference ($P < 0.0001$) between the two treatments, and the rate of accumulation increases with

increasing concentrations of arsenic. The result showed a significant difference ($P < 0.0001$) between the *P. vittata* inoculated with *Bacillus* sp. 3P20 and spiked with 200 and 500 mg/L arsenic, having a total arsenic content of 240 and 255.25 mg/kg, respectively, compared to the control (un-inoculated), which has 143 mg/kg. Although there was an increase in the arsenic content in the plants inoculated with 3P20 and spiked with 200 and 500 mg/L arsenic, no significant difference was observed across treatments. In contrast, there was a significant difference ($P < 0.0001$) in the total arsenic content of *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 200 mg/L and those spiked with 500 mg/L arsenic with the same isolate (Figure 5).

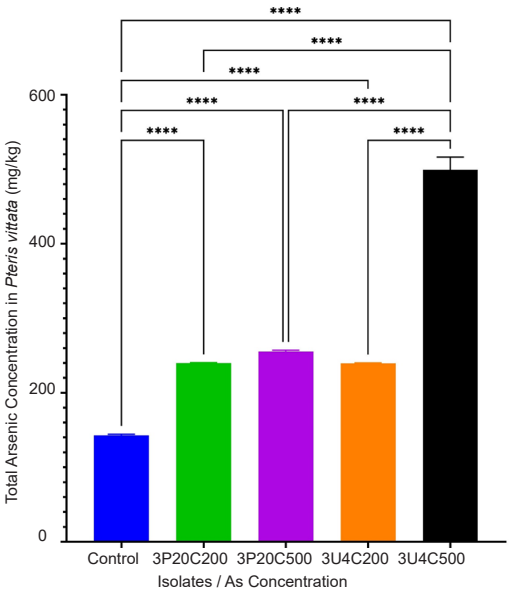



*Figure 5*. Total arsenic content of *Pteris vittata* after the eighth week of transplanting

*Note*. Data were analyzed using a one-way analysis of variance followed by Tukey's multiple comparisons tests. Data represents mean ± standard error of the mean (SEM); ****$P < 0.0001$ compared to control. 3P20 (*Bacillus* sp.) and 3U4 (*Enterobacter* sp.) are the bacterial isolates while C200 and C500 indicate 200 and 500 mg/L of arsenic concentrations, respectively

## Shoot and Root Dry Weights of *P. vittata*

After harvesting the plants, the dry shoot and root weights of *P. vittata* were taken. The results showed a significant decrease ($F_{4,15} = 81.33$, $P < 0.0001$) in the shoot dry weights of the *P. vittata* inoculated with *Bacillus* sp. 3P20 and spiked with 200 and 500 mg/L arsenic (2.27 and 2.53 g, respectively) compared to the control (3.35 g) at $P < 0.05$. Similarly, no significant difference was observed in *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 200 mg/L of arsenic with a dry shoot weight of 3.2 g, but there was a significant decrease in the shoot dry weights of *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 500 mg/L arsenic with the dry shoot weights (2.78 g), compared to the control shoot dry weight (3.35 g) (Figure 6a). The one-way ANOVA result revealed a significant increase ($F_{4,15} = 21.85$, $P < 0.0001$) in the root dry weights of *P. vittata* inoculated with all the isolates at different levels of arsenic. The root dry weight of the plant inoculated with *Bacillus* sp. 3P20 and spiked with 200 and 500 mg/L were observed to be 1.49 and 1.86 g, respectively, compared to the control (1.42 g). The root dry weights of *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 200 and 500 mg/L arsenic were observed to be 1.74 and 1.80 g, respectively. It showed a significant increase ($P < 0.0001$) in the root dry weight of the plant compared to the control (1.42 g) (Figure 6b).

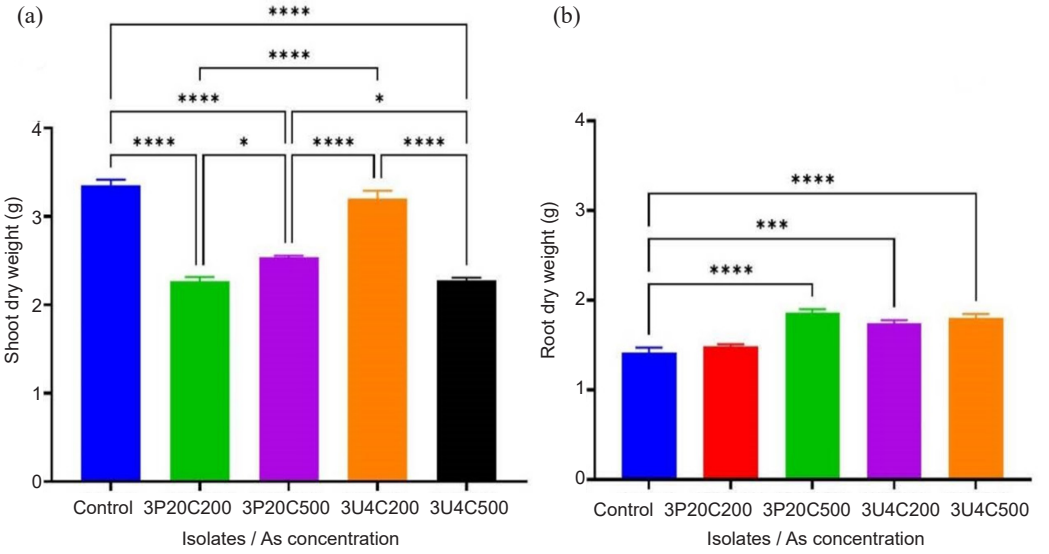



*Figure 6*. Shoots and roots dry weights of *Pteris vittata* after harvesting in the eighth week. (a) Shoot dry weight and (b) root dry weight

*Note*. Data were analyzed using a one-way analysis of variance followed by Tukey's multiple comparisons tests. Data represents mean ± standard error of the mean (SEM); *$P < 0.05$, ****$P < 0.0001$ compared to control; 3P20 (*Bacillus* sp.) and 3U4 (*Enterobacter* sp.) are the bacterial isolates while C200 and C500 indicate 200 and 500 mg/L of arsenic concentrations, respectively

## Chlorophyll Content (SPAD Values) of the *P. vittata*

The chlorophyll contents of the *P. vittata* were measured by non-destructive means (SPAD meter). The results of the SPAD values at the first, fourth and eighth week of transplanting are presented in Figures 7a-7c. The result showed there was no significant difference between the chlorophyll content of the plants inoculated with *Bacillus* sp. (3P20) and *Enterobacter* sp. (3U4) compared to the control at both the first and fourth week of transplanting (Figures 7a-7c). At the eighth week of transplanting, a two-way ANOVA indicated that there was a significant increase ($P < 0.005$) in the SPAD values of the *P. vittata* inoculated with 3P20 at 200 mg/L (34.23) and 500 mg/L arsenic (36.67) compared to the control plants (30.11) (Figure 7). On the other hand, there was a significant increase ($P < 0.05$) in the SPAD values of the *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 200 mg/L arsenic (36.45). However, there was no significant difference in the plant inoculated with the same isolate and spiked with 500 mg/L of arsenic (32.93) compared to the control (33.49). The results showed that the isolates significantly increased the chlorophyll content of the plants at the eighth week of transplanting ($P < 0.05$) (Figure 7). It could be due to the production of phytohormones and mineral solubilization, which are among the factors that help plant growth and development.

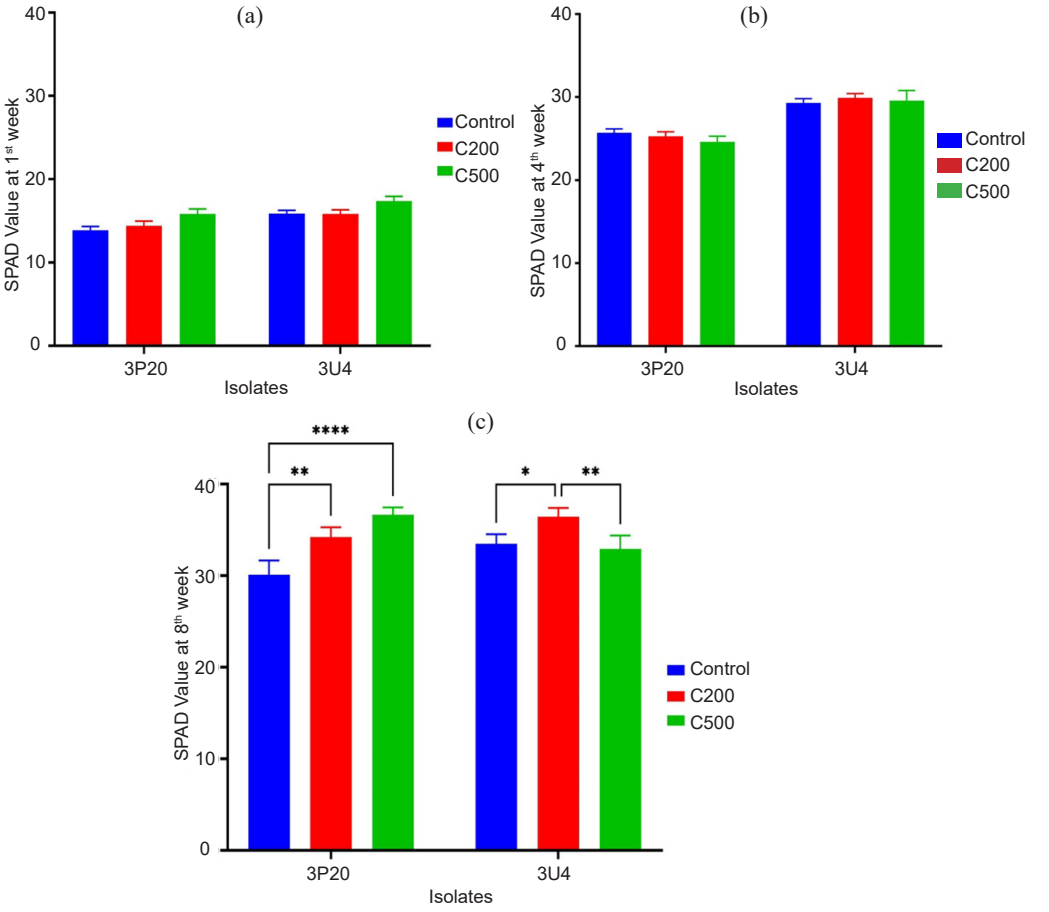



*Figure 7*. Chlorophyll content of *Pteris vittata* (a) first week, (b) fourth week, and (c) eighth week of transplanting, respectively

*Note*. Data were analyzed using a two-way analysis of variance followed by Tukey's multiple comparisons tests. Data represent mean ± standard error of the mean (SEM); *$P < 0.05$, **$P < 0.005$, ****$P < 0.0001$ compared to the control; 3P20 (*Bacillus* sp.) and 3U4 (*Enterobacter* sp.) are the bacterial isolates while C200 and C500 indicate 200 and 500 mg/L of arsenic concentrations, respectively

## Bioconcentration Factor of Arsenic in *P. vittata*

The BCF of arsenic in *P. vittata* was determined as presented below.

The BCF of arsenic in *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 500 mg/L arsenic solution $= \frac{499.5\ \text{mg/kg}}{5\ \text{mg/kg}} = 99.9$ ·

While BCF of arsenic in *P. vittata* inoculated with *Enterobacter* sp. 3U4 and spiked with 200 mg/L arsenic = $\frac{239.5\ \text{mg/kg}}{5\ \text{mg/kg}} = 47.9$ ·

The BCF of arsenic in *P. vittata* inoculated with *Bacillus* sp. 3P20 and spiked with 500 mg/L arsenic solution $= \frac{255.25\ \text{mg/kg}}{5\ \text{mg/kg}} = 51.05$ and BCF of arsenic

in *P. vittata* inoculated with *Bacillus* sp. 3P20 and spiked with 200 mg/L arsenic $= \frac{240\ \text{mg/kg}}{5\ \text{mg/kg}} = 48.$

## DISCUSSION

The SEM and TEM analyses revealed no significant changes in the morphology of the *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 cells when exposed to 500 mg/L of arsenic (As (III)). TEM was used to investigate the intracellular accumulation of arsenic. TEM results showed no significant changes between the control and the cells treated with 500 mg/L arsenic from this study. Ghosh et al. (2020) reported that the electron microscopy images displayed noticeable alterations in the morphology of *Bacillus* sp. strain IIIJ3-1 when exposed to arsenic stress in aerobic and anaerobic conditions. In their research, the cells treated with arsenic showed a reduction in size and a rugged and folded cell surface compared to the control cells (Ghosh et al., 2020). The TEM images of *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 showed no noticeable changes in the bacterial cells treated with 500 mg/L of arsenic compared to the control (untreated) cells. It was probably due to the ability of the bacteria to tolerate high concentrations of the toxic metal (up to 1,000 mg/L (*Bacillus* sp.) and 900 mg/L (*Enterobacter* sp.).

Arsenite-oxidizing bacteria can transform the toxic form of arsenite into the less harmful form of arsenate, while arsenate-reducing bacteria can convert arsenate back into arsenite (Liao et al., 2011). However, in both instances, a significant quantity of the converted arsenate and arsenite remains trapped within the bacterial cells instead of being released. The TEM study provided additional evidence by confirming the accumulation of arsenic inside the bacterial cells. Exposure to high-level arsenic can negatively impact the cell wall and cytoplasmic membrane of bacteria, resulting in compromised structural integrity and disrupted cellular functions.

Using arsenic-tolerant plant growth-promoting rhizobacteria (PGPR) in the bioremediation of arsenic-contaminated soils can be a promising strategy to mitigate the adverse effects of metal contamination on plants and the soil environment. These bacteria can assist in the growth and survival of plants in contaminated soils and improve the efficiency of phytoremediation. In arsenic-contaminated soils, phytohormones-producing microorganisms may stimulate plant growth and development (Lu et al., 2022; Q. Wang et al., 2019). The efficiency of arsenic phytoextraction can be enhanced by introducing naturally occurring bacteria with growth-promoting properties and arsenic-tolerant ability. It is especially beneficial for hyperaccumulator plant species such as *P. vittata*. These bacteria possess the ability to convert As (V) to As (III), leading to an overall improvement in the effectiveness of the arsenic remediation process (Lampis et al., 2015).

The inoculation of *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 in the soils influenced the ability of *P. vittata* to remove arsenic from the spiked soils. The bacteria also improved the phytoextraction ability

of *P. vittata*. Several hyperaccumulating plant species inoculated with rhizosphere or endophytic bacterial strains exhibited enhanced plant growth and biomass output (Sessitsch et al., 2013). A *Bacillus subtilis* strain demonstrated the ability to boost the growth of plants, leading to increased nickel accumulation. This improvement was likely due to the strain's IAA production ability, potentially promoting plant growth. Similarly, a chromium-resistant *Pseudomonas* strain that also produces IAA showed the ability to enhance the growth of *Brassica juncea* and, as a result, increased the extraction of trace elements.

*Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 produces plant growth-promoting substances such as phytohormones siderophores and solubilizes phosphate, potassium, and silicate, thereby promoting the growth and development of *P. vittata* even under arsenic stresses. The outcomes of this study showed that *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 could survive well in arsenic-contaminated soils and significantly enhance plant growth. Based on the results of the plant biomass, there was a decrease in the shoot dry weights of *P. vittata* despite the inoculation of the isolates, which could be due to the arsenic accumulation in the fronds of the plants. The excessive accumulation of arsenic hinders plant growth and biomass production by disrupting essential physiological processes like photosynthesis, respiration, and nutrient absorption (Han et al., 2020). Higher arsenic concentrations in its fronds are linked to reduced shoot dry weight (Kong et al., 2017). On the other hand, there was a significant increase in the root dry weights of the plants compared to the control (untreated), indicating an efficient translocation of the arsenic metal from the roots to the shoots because of bacterial inoculation.

The result showed a significant increase in the chlorophyll content of the plants at the eighth week of transplanting, which could be due to the production of phytohormones and mineral solubilization by the two bacterial strains, which are part of the factors that help plant growth and development. An essential condition for plant growth-promoting bacteria is the efficient colonization of rhizosphere soils by these bacteria (Q. Wang et al., 2018). *Pteris vittata* can withstand a total arsenic level of up to 5,000 mg/kg without experiencing any injury; as a result, the mechanism behind its resistance is of significant interest. Recent findings indicate enhanced ROS metabolism is a crucial physiological mechanism for arsenic resistance (Yan et al., 2019). The works of other researchers support these findings. Lampis et al. (2015) reported that a carefully selected mixture of bacteria, known for enhancing plant growth and aiding in arsenic transport, can improve arsenic extraction from contaminated environments. This technique has been successfully employed with the hyperaccumulator fern species *P. vittata*. The introduction of *Agrobacterium radiobacter* (strain D14) into *Populus deltoides* could enhance the tree's ability to tolerate arsenic, stimulate its growth, improve its absorption efficiency, and facilitate arsenic movement within the

plant (Q. Wang et al., 2011). When grown in arsenic-rich soil, *P. vittata* can extract arsenic through phytoextraction (Antenozio et al., 2021).

The capacity of *P. vittata* to endure and accumulate high levels of arsenic may be attributed, at least in part, to the presence of arsenic-tolerant bacteria that reside in symbiosis with the plant (Abou-Shanab et al., 2020; Cai et al., 2019). In a similar research, Xu et al. (2016) reported that arsenic-resistant endophytes could potentially facilitate plant growth in *P. vittata*, consequently enhancing phytoremediation efficiency in sites contaminated with arsenic. Diverse viewpoints exist concerning the precise location of arsenic conversion and the transportation of arsenite, which is the form of arsenic that moves from the roots to the fronds. It has been proposed that arsenate, rather than arsenite, is the primary form of arsenic transported from the roots to the fronds, and the conversion of arsenate primarily occurs in the fronds (Vandana et al., 2020). Q. Wang et al. (2018) reported that the survival of the greens growing in the contaminated soils was boosted by the inoculation of *Bacillus megaterium* bacteria (strain H3). Greens inoculated with strain H3 had an increase in edible tissue biomass compared to the controls. Bacteria can reduce the accumulation of metals in plant tissues and immobilize them in the soil. However, the specific impact of bacteria from the same genus on metal accumulation and immobilization variations remains unclear. It is evident that different types of PGPR assist in the remediation of soil contaminated with trace metals through diverse mechanisms (Guo et al., 2020).

Plant-microbe interactions play a significant role in plant development, transport, and uptake of nutrients in the rhizosphere. Many studies have demonstrated that the microbiota in a plant's environment directly affects how well it responds to local environmental stress (Upadhyay et al., 2018). Several bacterial genera, including *Acidovorax*, *Alcaligenes*, *Bacillus*, *Mycobacterium*, *Paenibacillus*, *Pseudomonas*, and *Rhodococcus*, have been frequently used in phytoremediation (Sharma, 2021). Bacteria have proven useful in phytoremediation by increasing metal bioavailability by synthesizing organic acids, carbohydrates, and plant growth stimulants. Thus, the effect of phytoremediation might be influenced by all of the elements that influence plant root development and productivity, such as light, temperature, humidity, and soil properties (Liu et al., 2017; Sharma, 2021). The effects of phytoremediation by bacteria, primarily *Bacillus* and *Pseudomonas* species, are directly influenced by plant development and biomass. It can improve phytoremediation by promoting plant growth (Sharma et al., 2021). The combined inoculations of local bacteria can boost arsenic accumulation and *P. vittata* biomass, lowering the soil's arsenic content (Antenozio et al., 2021).

Most of the rhizosphere bacteria identified from arsenic-rich and natural mineral soils can both oxidize As (III) to As (V) or reduce As (V) to As (III). The inoculation of *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 in the soils influenced the ability of *P. vittata* to remove arsenic from the spiked soils. The microbes

supplied PGP substances such as IAA, phytohormones that promote plant growth, and siderophores that increase iron uptake and are observed to solubilize phosphate and potassium. The bacteria also improved the phytoextraction ability of *P. vittata*. Several hyperaccumulating plant species inoculated with rhizosphere or endophytic bacterial strains exhibited enhanced plant growth and biomass output (Sessitsch et al., 2013).

The BCF of arsenic in *P. vittata* across treatments was greater than 1, indicating its suitability for arsenic bioremediation. Plants with a BCF value exceeding one are viable candidates for phytoextraction (Debela et al., 2022).

## CONCLUSION

The inoculation of *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 in the soils spiked with 200 and 500 mg/L arsenic significantly influences the arsenic hyperaccumulation ability of *P. vittata*. The results indicate a significant increase in the growth of the plants, and the rate of hyperaccumulation of the arsenic metal increases in inoculated plants compared to the control (untreated) *P. vittata*. Based on the result, the isolates play a significant role in arsenic biotransformation, its hyperaccumulation by *P. vittata*, and its survival and growth in the contaminated soils.

## ACKNOWLEDGMENTS

The authors express their appreciation to Universiti Sains Malaysia for providing necessary research facilities and to the Ministry of Higher Education Malaysia for financial support under the Fundamental Research Grant Scheme (FRGS), Project Code FRGS/1/2019/STG03/USM/02/7.

## REFERENCES

Abou-Shanab, R. A. I., Mathai, P. P., Santelli, C., & Sadowsky, M. J. (2020). Indigenous soil bacteria and the hyperaccumulator *Pteris vittata* mediate phytoremediation of soil contaminated with arsenic species. *Ecotoxicology and Environmental Safety*, *195*, 110458. https://doi.org/10.1016/j.ecoenv.2020.110458

Alka, S., Shahir, S., Ibrahim, N., Chai, T.-T., Bahari, Z. M., & Manan, F. A. (2020). The role of plant growth promoting bacteria on arsenic removal: A review of existing perspectives. *Environmental Technology and Innovation*, *17*, 100602. https://doi.org/10.1016/j.eti.2020.100602

Antenozio, M. L., Giannelli, G., Marabottini, R., Brunetti, P., Allevato, E., Marzi, D., Capobianco, G., Bonifazi, G., Serranti, S., Visioli, G., Stazi, S. R., & Cardarelli, M. (2021). Phytoextraction efficiency of *Pteris vittata* grown on a naturally As-rich soil and characterization of As-resistant rhizosphere bacteria. *Scientific Reports*, *11*, 6794. https://doi.org/10.1038/s41598-021-86076-7

Bui, T. K. A. (2017). Phytoremediation potential of *Pteris vittata* L. and *Eleusine indica* L. through field study and greenhouse experiments. *Journal of Vietnamese Environment*, *8*(3), 156–160. https://doi.org/10.13141/jve.vol8.no3.pp156-160

Cai, C., Lanman, N. A., Withers, K. A., DeLeon, A. M., Wu, Q., Gribskov, M., Salt, D. E., & Banks, J. A. (2019). Three genes define a bacterial-like arsenic tolerance mechanism in the arsenic hyperaccumulating fern *Pteris vittata*. *Current Biology*, *29*(10), 1625-1633.e3. https://doi.org/10.1016/j.cub.2019.04.029

Debela, A. S., Dawit, M., Tekere, M., & Itanna, F. (2022). Phytoremediation of soils contaminated by lead and cadmium in Ethiopia, using Endod (*Phytolacca dodecandra* L). *International*

*Journal of Phytoremediation*, *24*(13), 1339–1349. https://doi.org/10.1080/15226514.2021.2025336

Ghosh, S., Mohapatra, B., Satyanarayana, T., & Sar, P. (2020). Molecular and taxonomic characterization of arsenic (As) transforming *Bacillus* sp. strain IIIJ3-1 isolated from As-contaminated groundwater of Brahmaputra river basin, India. *BMC Microbiology*, *20*, 256. https://doi.org/10.1186/s12866-020-01893-6

Guo, J., Muhammad, H., Lv, X., Wei, T., Ren, X., Jia, H., Atif, S., & Hua, L. (2020). Chemosphere Prospects and applications of plant growth promoting rhizobacteria to mitigate soil metal contamination: A review. *Chemosphere*, *246*, 125823. https://doi.org/10.1016/j.chemosphere.2020.125823

Han, Y.-H., Jia, M.-R., Wang, S.-S., Deng, J.-C., Shi, X.-X., Chen, D.-L., Chen, Y., & Ma, L. Q. (2020). Arsenic accumulation and distribution in *Pteris vittata* fronds of different maturity: Impacts of soil As concentrations. *Science of the Total Environment*, *715*, 135298. https://doi.org/10.1016/j.scitotenv.2019.135298

Kong, Z., Deng, Z., Glick, B. R., Wei, G., & Chou, M. (2017). A nodule endophytic plant growth-promoting *Pseudomonas* and its effects on growth, nodulation and metal uptake in *Medicago lupulina* under copper stress. *Annals of Microbiology*, *67*, 49–58. https://doi.org/10.1007/s13213-016-1235-1

Lampis, S., Santi, C., Ciurli, A., Andreolli, M., & Vallini, G. (2015). Promotion of arsenic phytoextraction efficiency in the fern *Pteris vittata* by the inoculation of As-resistant bacteria: A soil bioremediation perspective. *Frontiers in Plant Science*, *6*, 80. https://doi.org/10.3389/fpls.2015.00080

Liao, V. H.-C., Chu, Y.-J., Su, Y.-C., Hsiao, S.-Y., Wei, C.-C., Liu, C.-W., Liao, C.-M., Shen, W.-C., & Chang, F.-J. (2011). Arsenite-oxidizing and arsenate-reducing bacteria associated with arsenic-rich groundwater in Taiwan. *Journal of Contaminant Hydrology*, *123*(1–2), 20–29. https://doi.org/10.1016/j.jconhyd.2010.12.003

Liu, S.-H., Zeng, G.-M., Niu, Q.-Y., Liu, Y., Zhou, L., Jiang, L.-H., Tan, X.-F., Xu, P., Zhang, C., & Cheng, M. (2017). Bioremediation mechanisms of combined pollution of PAHs and heavy metals by bacteria and fungi: A mini review. *Bioresource Technology*, *224*, 25–33. https://doi.org/10.1016/j.biortech.2016.11.095

Lu, Y., Liao, S., Ding, Y., He, Y., Gao, Z., Song, D., Tian, W., & Zhang, X. (2022). Effect of *Stevia rebaudiana* Bertoni residue on the arsenic phytoextraction efficiency of *Pteris vittata* L. *Journal of Hazardous Materials*, *421*, 126678. https://doi.org/10.1016/j.jhazmat.2021.126678

Manzoor, M., Abid, R., Rathinasabapathi, B., De Oliveira, L. M., da Silva, E., Deng, F., Rensing, C., Arshad, M., Gul, I., Xiang, P., & Ma, L. Q. (2019). Metal tolerance of arsenic-resistant bacteria and their ability to promote plant growth of *Pteris vittata* in Pb-contaminated soil. *Science of the Total Environment*, *660*, 18–24. https://doi.org/10.1016/j.scitotenv.2019.01.013

Muazu A. S. (2024). *Bacterial population of* Pteris vittata *with potentials for bioremediation of arsenic rich soils and plant growth promotion* [Unpublished Doctoral thesis]. Universiti Sains Malaysia.

Nacoon, S., Jogloy, S., Riddech, N., Mongkolthanaruk, W., Kuyper, T. W., & Boonlue, S. (2020). Interaction between phosphate solubilizing bacteria and arbuscular mycorrhizal fungi on growth promotion and tuber inulin content of *Helianthus tuberosus* L. *Scientific Reports*, *10*, 4916. https://doi.org/10.1038/s41598-020-61846-x

Popov, M., Zemanová, V., Sácký, J., Pavlík, M., Leonhardt, T., Matoušek, T., Kaňa, A., Pavlíková, D., & Kotrba, P. (2021). Arsenic accumulation and speciation in two cultivars of *Pteris cretica* L. and characterization of arsenate reductase Pc*ACR2* and arsenite transporter Pc*ACR3* genes in the hyperaccumulating cv. Albo-

lineata. *Ecotoxicology and Environmental Safety*, *216*, 112196. https://doi.org/10.1016/j.ecoenv.2021.112196

Sessitsch, A., Kuffner, M., Kidd, P., Vangronsveld, J., Wenzel, W. W., Fallmann, K., & Puschenreiter, M. (2013). The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. *Soil Biology and Biochemistry*, *60*, 182–194. https://doi.org/10.1016/j.soilbio.2013.01.012

Setyawan, H. B., Yulianto, R., Santoso, W. D., & Suryandari, N. (2021). Fern plant (*Pteris vittata*) as a phytoremediator of arsenic heavy metal and its effect to the growth and quality of Kale (*Ipomea reptans* Poir). In *IOP Conference Series: Earth and Environmental Science* (Vol. 637, No. 1, p. 012066). IOP Publishing. https://doi.org/10.1088/1755-1315/637/1/012066

Sharma, P. (2021). Efficiency of bacteria and bacterial assisted phytoremediation of heavy metals: An update. *Bioresource Technology*, *328*, 124835. https://doi.org/10.1016/j.biortech.2021.124835

Sharma, P., Tripathi, S., Chaturvedi, P., Chaurasia, D., & Chandra, R. (2021). Newly isolated *Bacillus* sp. PS-6 assisted phytoremediation of heavy metals using *Phragmites communis*: Potential application in wastewater treatment. *Bioresource Technology*, *320*(Part B), 124353. https://doi.org/10.1016/j.biortech.2020.124353

Tirry, N., Tahri Joutey, N., Sayel, H., Kouchou, A., Bahafid, W., Asri, M., & El Ghachtouli, N. (2018). Screening of plant growth promoting traits in heavy metals resistant bacteria: Prospects in phytoremediation. *Journal of Genetic Engineering and Biotechnology*, *16*(2), 613–619. https://doi.org/10.1016/j.jgeb.2018.06.004

Tiwari, S., Sarangi, B. K., & Thul, S. T. (2016). Identification of arsenic resistant endophytic bacteria from *Pteris vittata* roots and characterization for arsenic remediation application. *Journal of Environmental Management*, *180*, 359–365. https://doi.org/10.1016/j.jenvman.2016.05.029

Upadhyay, M. K., Yadav, P., Shukla, A., & Srivastava, S. (2018). Utilizing the potential of microorganisms for managing arsenic contamination: A feasible and sustainable approach. *Frontiers in Environmental Science*, *6*, 24. https://doi.org/10.3389/fenvs.2018.00024

Vandana, U. K., Gulzar, A. B. M., Singha, L. P., Bhattacharjee, A., Mazumder, P. B., & Pandey, P. (2020). Hyperaccumulation of arsenic by *Pteris vittata*, a potential strategy for phytoremediation of arsenic-contaminated soil. *Environmental Sustainability*, *3*, 169–178. https://doi.org/10.1007/s42398-020-00106-0

Wang, J., Zhao, F. J., Meharg, A. A., Raab, A., Feldmann, J., & Mcgrath, S. P. (2002). Mechanisms of arsenic hyperaccumulation in *Pteris vittata*. Uptake kinetics, interactions with phosphate, and arsenic speciation. *Plant Physiology*, *130*(3), 1552-1561. https://doi.org/10.1104/pp.008185

Wang, Q., Ma, L., Zhou, Q., Chen, B., Zhang, X., Wu, Y., Pan, F., Huang, L., Yang, X., & Feng, Y. (2019). Inoculation of plant growth promoting bacteria from hyperaccumulator facilitated non-host root development and provided promising agents for elevated phytoremediation efficiency. *Chemosphere*, *234*, 769–776. https://doi.org/10.1016/j.chemosphere.2019.06.132

Wang, Q., Xiong, D., Zhao, P., Yu, X., Tu, B., & Wang, G. (2011). Effect of applying an arsenic-resistant and plant growth-promoting rhizobacterium to enhance soil arsenic phytoremediation by *Populus deltoides* LH05-17. *Journal of Applied Microbiology*, *111*(5), 1065–1074. https://doi.org/10.1111/j.1365-2672.2011.05142.x

Wang, Q., Zhang, W.-J., He, L.-Y., & Sheng, X.-F. (2018). Increased biomass and quality and reduced heavy metal accumulation of edible tissues of vegetables in the presence of Cd-tolerant and immobilizing *Bacillus megaterium* H3. *Ecotoxicology and Environmental Safety*, *148*, 269–274. https://doi.org/10.1016/j.ecoenv.2017.10.036

Xu, J.-Y., Han, Y.-H., Chen, Y., Zhu, L.-J., & Ma, L. Q. (2016). Arsenic transformation and plant growth promotion characteristics of As-resistant endophytic bacteria from As-hyperaccumulator *Pteris vittata*. *Chemosphere*, *144*, 1233–1240. https://doi.org/10.1016/j.chemosphere.2015.09.102

Yan, H., Gao, Y., Wu, L., Wang, L., Zhang, T., Dai, C., Xu, W., Feng, L., Ma, M., Zhu, Y.-G., & He, Z. (2019). Potential use of the *Pteris vittata* arsenic hyperaccumulation-regulation network for phytoremediation. *Journal of Hazardous Materials*, *368*, 386–396. https://doi.org/10.1016/j.jhazmat.2019.01.072

Yang, C., Han, N., Inoue, C., Yang, Y.-L., Nojiri, H., Ho, Y.-N., & Chien, M.-F. (2022). Rhizospheric plant-microbe synergistic interactions achieve efficient arsenic phytoextraction by *Pteris vittata*. *Journal of Hazardous Materials*, *434*, 128870. https://doi.org/10.1016/j.jhazmat.2022.128870

Pertanika J. Trop. Agri. Sci. 47 (4): 1379 - 1389 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

*Short Communication*

# *In-vivo* Toxicity Assessment of the Garlic Juice Extract (*Allium sativum*) in Juvenile Hybrid Grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*)

**Muhamad Izzuan-Razali[1,2], Mohd Firdaus-Nawi[1]*, Shaharah Mohd Idris[2], Azila Abdullah[3], Nik Haiha Nik Yusoff[2], Rimatulhana Ramly[3], Mohd Syafiq Mohammad Ridzuan[1,3], Sufian Mustafa[1,2] and Rashidah Abdul Razak[1]**

[1]*Aquatic Microbiology Research Laboratory, Institute of Oceanography and Maritime Studies, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia*
[2]*Marine Fishes Aquaculture Research Division, Fisheries Research Institute, Tanjung Demong, 22200 Besut, Terengganu, Malaysia*
[3]*National Fish Health Research Division, Fisheries Research Institute, 11960 Batu Maung, Penang, Malaysia*

## ABSTRACT

The toxicity of garlic juice extract in juvenile hybrid grouper was evaluated via bath and oral administration. A total of 280 fish, each with an average weight of 20 ± 5 g, were evenly distributed among 28 glass aquaria. This distribution was designed to represent seven test concentration groups, each implemented in duplicate. The fish were immersed in freshly prepared garlic juice extracts at 0, 500, 600, 700, 800, 900, and 1,000 ppm concentrations. Meanwhile, pellets containing 0, 20, 40, 60, 80, and 100% garlic juice extract were administered for oral exposure. The median lethal concentration of garlic juice extract following bath immersion was recorded at 993.11 ppm after 96 hr. Besides, there was no mortality in all groups exposed to garlic juice extract orally, indicating that the extract has a shallow effect on juvenile hybrid groupers when ingested.

*Keywords*: *Allium sativum*, fish, garlic, hybrid grouper, toxicity

ARTICLE INFO

*Article history:*
Received: 03 November 2023
Accepted: 23 February 2024
Published: 29 November 2024

DOI: https://doi.org/10.47836/pjtas.47.4.19

E-mail addresses:
muhamadizzuan17@gmail.com (Muhamad Izzuan-Razali)
firdausn@iium.edu.my (Mohd Firdaus-Nawi)
shaharah@dof.gov.my (Shaharah Mohd Idris)
azila@dof.gov.my (Azila Abdullah)
nhaiha1960@gmail.com (Nik Haiha Nik Yusoff)
rimatulhana@dof.gov.my (Rimatulhana Ramly)
syafiq@dof.gov.my (Mohd Syafiq Mohammad Ridzuan)
sufnor96@yahoo.com (Sufian Mustafa)
rashidahrazak@iium.edu.my (Rashidah Abdul Razak)
*Corresponding author

## INTRODUCTION

Garlic (*Allium sativum*) belongs to the Alliaceae family, with approximately 600 identified species of *Allium*. Its native range includes Europe, North America,

ISSN: 1511-3701
e-ISSN: 2231-8542

North Africa, and Asia (Chen et al., 2019). Garlic has medical properties in addition to being widely used in cooking as a spicy and nutty flavour profile. Moreover, the predated ancient Egypt used it to treat illnesses among pyramid labourers, and it was also utilised for healing by the Babylonians, Greeks, and Romans (Koch & Lawson, 1996). In his earliest work in 1958, Louis Pasteur provided evidence of garlic's antibacterial properties (Bayan et al., 2014). Garlic extracts effectively treat stomach-related illnesses caused by common pathogenic intestinal bacteria and protozoan parasite *Entamoeba histolytica* (Shasany et al., 2000). Garlic oil inhalations are beneficial against coughs, red eyes, and impotence (Farid et al., 2022). Additionally, garlic has been shown to have anti-cancer effects and to reduce cardiovascular risk through its cholesterol-regulating properties (Zhang et al., 2020).

Petropoulos et al. (2018) previously reported that garlic bulbs are nutritionally dense in carbs and proteins. Garlic contains 33 sulphur compounds, 17 amino acids, many enzymes, and minerals. Its pungent odour is derived from alliin, a sulphur-containing molecule. When garlic is crushed or sliced, alliin (S-allyl cysteine sulfoxide) will be converted to allicin upon activating the alliinase enzyme (diallyl disulfide). Previous research has shown that allicin, a volatile sulphur compound, is responsible for a variety of intriguing activities such as antimicrobial, antioxidant, antihelminthic, immunostimulant, growth, and appetite stimulator (Fridman et al., 2014; Guo et al., 2015; Syngai et al., 2016).

A toxicity test is required before certain chemicals or treatments are intended for living organisms. Toxicity testing was described by Arome and Chinedu (2013) as evaluating and classifying the potential risks associated with a given test material. Acute aquatic toxicity would usually be determined using a median lethal concentration 96-hr ($LC_{50}$-96 hr) test, which quantifies the dose or amount of test compound capable of causing 50% mortality in a population following 96 hr of exposure (Islam et al., 2021). Various exposure modes, including oral and cutaneous routes, are utilised to determine the appropriate dosage for long-term toxicity studies (Strickland et al., 2018). A probable toxic effect can be detected through behavioural anomalies, typically reflected as a sign of damage to the nervous system upon exposure (Almeida et al., 2009).

The hybrid grouper is one of Malaysia's most cultivated marine fish species (Ridzuan et al., 2022), with a cumulative production of 3,871.88 metric tonnes in 2022 (Department of Fisheries [DOF], 2022). The species results from a crossbreeding program held by the Borneo Marine Research Institute (BMRI) and collaborators. Since then, the fish has gained popularity in the mariculture industry, most notably in Southeast Asia. The hybrid grouper is relatively easy to handle, has a high salinity and pH tolerance, and, perhaps above all, grows rapidly (Sutthinon et al., 2014). However, hybrid groupers, similar to other grouper species, are particularly prone to vibriosis, which results in severe mortality, especially at

the juvenile stage (Mohamad et al., 2019). Utilising a medicinal plant extract with proven antibacterial activity may reduce the impact. A previous study by Mercy and Gopalakannan (2018) reported that the aqueous preparation of garlic extract resulted in a remarkable inhibition of *Vibrio* sp. isolated from shrimp farms and, therefore, could be used to combat vibriosis. Valenzuela-Gutiérrez et al. (2021) reported multiple properties of garlic, including antibacterial and has been used in various aquaculture fish species such as tilapia, rainbow trout, spotted grouper, catfish and barramundi

However, studies on the toxicity effect of garlic juice extract in marine fish, especially hybrid grouper, are scarce. Hence, this paper reports a $LC_{50}$-96 hr of garlic (*A. sativum* L.) juice extract in juvenile hybrid grouper following bath immersion and oral ingestion exposure.

## MATERIALS AND METHODS

### Animal Ethics

The procedures for handling fish have adhered to the guidelines established by the Institutional Animal Care and Use Committee (IACUC), International Islamic University of Malaysia (IIUM), as certified in IACUC-2019-31.

### Experimental Animals

The study procured healthy juvenile hybrid grouper with an average weight of 20 ± 5 g from the Marine Fishes Aquaculture Research Division, Fisheries Research Institute (FRI), Tanjung Demong, Terengganu, Malaysia. These fish were acclimatised in a 1 $m^3$ fibreglass tank. Subsequently, ten fish were slain and screened for bacterial, viral, and parasitic diseases. If any pathogen is identified, a treatment procedure will be implemented to ensure all the experimental fish are disease-free at the commencement of the trial. The fish were fed *ad libitum* with commercial feed, and the water was kept aerated continuously. Daily, the water was fully exchanged, and water quality parameters, including salinity, temperature, pH, and dissolved oxygen (DO), were determined with a handheld ProQuatro multiparameter (YSI, USA).

### Extraction of Garlic Juice Extract

Three kilograms of fresh garlic (*A. sativum*) were purchased locally. The juice was extracted using an extractor by crushing peeled garlic bulbs. The stock extract was then subjected to filtration through a muslin cloth and subsequently stored in hermetically sealed bottles at 4°C until it was utilised. The juice collected was regarded as 100% garlic juice extract.

### Preparation of Garlic Juice Extract for Bath Immersion Treatment

The freshly prepared garlic juice extract was diluted in seawater to achieve the desired concentrations of 500, 600, 700, 800, 900, and 1,000 ppm, following the methodology outlined by Yunus et al. (2019) with slight modifications.

**Preparation of Garlic Juice Extract for Oral Exposure**

The garlic juice extract was diluted in ethanol to achieve concentrations of 20, 40, 60, 80, and 100%, and subsequently sprayed onto fish pellets at a 50 ml/kg ratio and allowed to dry at room temperature with continuous airflow (D.-H. Lee et al., 2012; Syngai et al., 2016).

**Experimental Design**

The acute toxicity tests, aimed at evaluating the $LC_{50}$-96 hr exposure by bath immersion, were carried out according to Yunus et al. (2019) with slight modifications. For a 96-hr exposure period, a static renewal bioassay system with constant aeration was established, consisting of six groups of garlic juice concentration gradients and one untreated control. Following a two-week acclimatisation period, 280 juvenile hybrid groupers were divided evenly in each test concentration of 500, 600, 700, 800, 900, and 1,000 ppm, along with an untreated control group glass aquarium (50 cm x 40 cm x 50 cm) in duplicate filled with 50 L of seawater. The untreated group received no garlic extract addition, which served as a negative control. Water quality parameters, including salinity, temperature, pH, and DO, were routinely monitored throughout the study using a ProQuatro multiparameter (YSI, USA). At a predetermined time each day, full (100%) water was exchanged, and test solutions were replenished to preserve the desirable concentration of test extract. Fish were fed twice daily with a total of 2% of the average body weight, with commercial feed containing 44% crude protein (SeaMaster, Vietnam). The behaviour of fish was thoroughly observed, and the number of mortalities was recorded at 24-, 48-, 72-, and 96-hr intervals.

Similarly, a static system was employed in the acute toxicity testing via oral feeding, comprising five groups of garlic juice concentration gradients and one untreated control. In this setup, 20 fish were divided in duplicate into each test concentration of 20, 40, 60, 80, and 100%, and the untreated control group glass aquarium filled with 50 L of seawater. Each treated group was fed twice daily with a total of up to 2% of the average body weight with commercial feed containing 44% crude protein (SeaMaster, Vietnam) added with a respective concentration of garlic juice extract, whereas the untreated negative control fish were fed with commercial pellets without garlic juice extract. At a predetermined time each day, 100% water was exchanged, and the behaviour of the fish was thoroughly observed. Mortalities were documented at 24-, 48-, 72-, and 96-hr intervals.

**Data Analysis**

The results are expressed as the mean ± SD. The DO statistical significance was evaluated using one-way analysis of variance (ANOVA), and means were separated by Tukey's test using SPSS (version 25). Significant differences were considered at $p<0.05$. A curve fitting was performed using an online tool, MyCurveFit, to calculate the median lethal concentration ($LC_{50}$) values.

## RESULTS

### Determination of $LC_{50}$ by Bath Immersion

Table 1 shows the mortality rate of juvenile hybrid grouper exposed to various doses of garlic juice extracts. Initial mortality was only observed on day 2 (2.5%) and day 3 (2.5%) in the group of fish immersed in 900 and 700 ppm of garlic juice extract, respectively. However, on day 4 post-treatment, there was widespread mortality, with 23 fish deaths (57.5%) in the group immersed in 1,000 ppm garlic juice extract. In contrast, there was no mortality in the untreated control groups, 500, and 600 ppm groups, during the 96-hr experiment. As a result, the $LC_{50}$ of juvenile hybrid grouper following bath immersion in garlic juice extract was calculated at 993.11 ppm (Figure 1).

Figure 2 summarises the DO value in all tested groups following the 96-hr trial. At the commencement of the experiment, there was no significant difference ($p>0.05$) in DO levels across the groups. However, after 24 hr of bath immersion exposure, the DO levels in the 600, 700, 800, 900, and 1,000 ppm treatment groups decreased significantly ($p<0.05$). This downward trend persisted until the end of the experiment. The lowest DO level was recorded at $5.7 \pm 0.04$ mg/L in the 1,000-ppm group at 72 hr post-exposure. Meanwhile, the control and 500 ppm groups showed no significant decrease in DO ($p>0.05$) throughout the experiment. The fish exposed to various concentrations of garlic juice extract exhibited changes in behaviour. In the first 24-hr period, the fish in the group exposed to 700, 800, 900, and 1,000 ppm became less aggressive. However, the fish in the negative control group exposed to low doses (500 and 600 ppm) showed normal behaviour. After 72 hr, fish exposed to the highest concentrations (1,000 ppm) increased their opercula activity and gasped for air at the water surface. The fish also exhibit anomalous behaviour, including lethargy, being less attentive, having a pale body, and sometimes swimming erratically.

### Determination of $LC_{50}$ by Oral Treatment

After being fed a commercial pellet containing various concentrations of garlic juice extract (20, 40, 60, 80, and 100%), no mortality was encountered in any treatment groups and untreated control. Additionally, the experimental fish remained active and did not exhibit clinical signs or visible abnormalities consistent with toxicity. However, there were observations of fish at high concentrations (900 and 1,000 ppm) experiencing instances of regurgitation of a small number of fish feed during the experiment. Yet, they continued to exhibit normal levels of activity. Consequently, the determination of the $LC_{50}$ was not feasible. Figure 3 presents DO levels in all tested groups following the 96-hr trial. There was no significant difference ($p>0.05$) in DO levels between treated and untreated groups.

Table 1
*Mortality rate within 96 hr of bath immersion exposure to varied doses of garlic juice extract*

| Group (ppm) \ Time (hr) | 12 | 24 | 48 | 72 | 96 | Number of mortality | Percentage (%) |
|---|---|---|---|---|---|---|---|
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 500 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 600 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 700 | 0 | 0 | 0 | 1 | 1 | 2 | 5 |
| 800 | 0 | 0 | 0 | 0 | 1 | 1 | 2.5 |
| 900 | 0 | 1 | 0 | 0 | 2 | 3 | 7.5 |
| 1,000 | 0 | 0 | 0 | 0 | 23 | 23 | 57.5 |

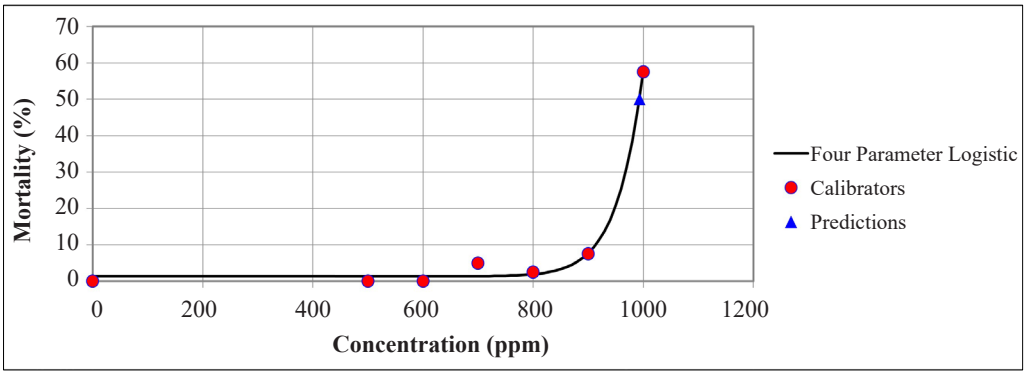



*Figure 1*. The median lethal concentration of garlic juice extract was calculated at 993.11 ppm following bath immersion within 96 hr

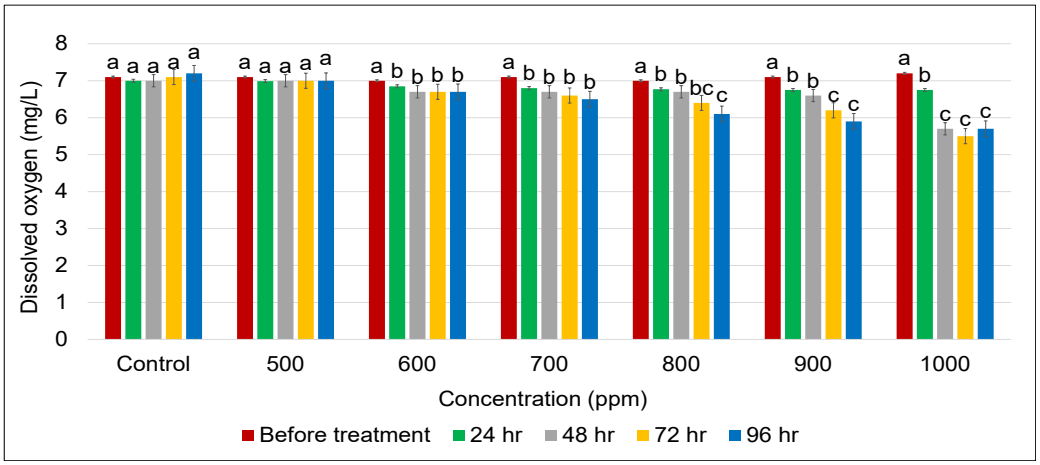



*Figure 2*. Values of dissolved oxygen in the seawater added with 500, 600, 700, 800, 900, and 1,000 ppm garlic juice extract. No garlic juice extract was added to the negative control group (Control)

*Note*. Different superscripts indicate a significant difference ($p$<0.05)

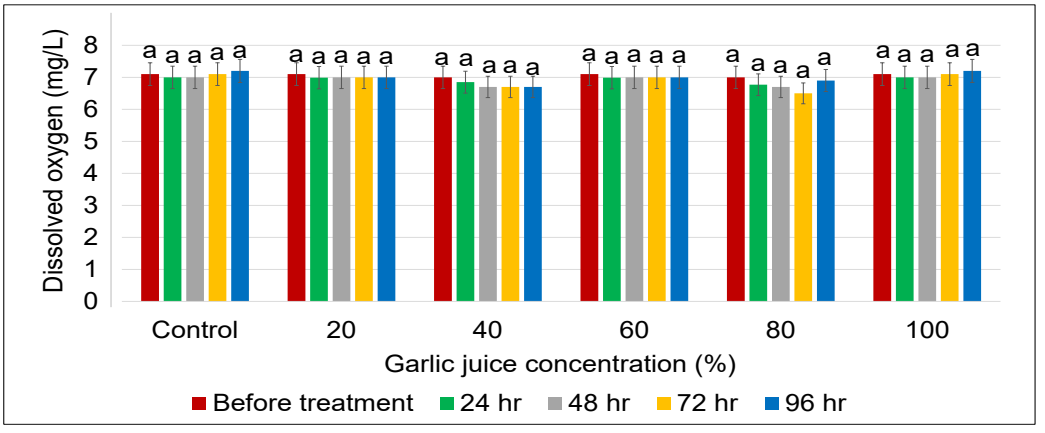



*Figure 3*. Values of dissolved oxygen in the seawater without adding garlic juice extract. However, juvenile hybrid groupers in those glass aquaria were fed commercial pellets with 500, 600, 700, 800, 900, and 1,000 ppm garlic juice extract. Juvenile hybrid groupers in the negative control group (Control) were fed with commercial pellet without garlic juice extract.

*Note*. Different superscripts indicate a significant difference ($p$<0.05)

## DISCUSSION

The present study evaluates the acute toxicity of garlic juice extract in juvenile hybrid grouper by determining the $LC_{50}$, which represents the concentration of extract that caused 50% mortality after a continuous 96-hr exposure period. Therefore, the higher $LC_{50}$ values reflect lower toxicity of the test substance, as greater concentrations are required to kill 50% of total organisms (Erhirhie et al., 2018). After bath immersion and exposure to garlic juice extract, this study obtained a high $LC_{50}$ value of 993.11 ppm. Meanwhile, deaths occur in fish groups exposed to juice extract ranging from 700 to 1,000 ppm after 72 hr. This finding suggested that the susceptibility of the fish to the extract's toxicity effects was concentration- and time-dependent, with mortality rising as both factors increased.

It is believed that the cause of death was due to oxidised sulphur compounds toxicity because the DO level in all treatment groups was above 4 mg/L, the minimum requirement for grouper rearing (Nasukha et al., 2021). Allicin and other sulphur compounds in garlic juice extract are susceptible to oxidation, especially in the presence of water. The high dissolved oxygen in the water will react with these sulphur compounds, forming various sulphur-containing oxides such as sulphur dioxide ($SO_2$) and sulphur trioxide ($SO_3$). Both compounds are toxic and cause severe irritations to the respiratory tract, skin, and mucous membranes (Agency for Toxic Substances and Disease Registry [ATSDR], 2023a, 2023b). The operculum of the dead fish was wide open, indicating that the fish was battling for oxygen. This condition might be due to the damaged respiratory system from the toxicity of oxidised sulphur compounds. The higher concentration of garlic juice extract in the water will result

in a lower dissolved oxygen concentration due to the oxygen-sulphur reaction. The phenomenon was observed in the 1,000-ppm immersion group, where the significantly lowest concentration of DO was recorded. The lowest concentration of DO in the water indicated the highest concentration of toxic oxidised sulphur compounds in the water, which led to the highest mortality rate. The dead fish's body also turns pale and noticeably discoloured, owing to the garlic's burning effect and low oxygen level. A similar study indicated that the depigmentation of the fish body under toxic stress is associated with endocrine gland dysfunction (Herrera et al., 2019).

In contrast, the garlic-enriched feed ingests are exceptionally safe for juvenile hybrid grouper. After being fed with a varied concentration of garlic juice extracts, no mortality was encountered in any treatment groups. Furthermore, throughout the experimental trial, all fish remained highly active and showed no visible signs of toxicity. Therefore, the $LC_{50}$ of fish fed with a commercial pellet containing varying percentages of garlic juice extract could not be determined. Garlic juice has been widely employed in other farmed animals for various purposes. As such, garlic aids in the digestion, absorption, and retention of nitrogen in cattle (Wanapat et al., 2008). Previous studies by Foysal et al. (2019) indicated that a variety of organosulfur compounds in garlic, such as S-methyl cysteine sulfoxide, diallyl disulfide, sallycysteine, ajoene, and allicin garlic, improve the performance tilapia's gut microflora which subsequently increases digestion, energy consumption, and promotes their growth. However, supplementing garlic as a feed additive should be done with caution. High concentrations of this plant extract may impair fish growth by facilitating excessive alkyl sulphide into the intestine, which could interfere with normal metabolism (J.-Y. Lee & Gao, 2012).

Assessing the toxicity of garlic juice extract across different fish species is vitally important for understanding its potential biological impact. Certain species might be more sensitive to garlic's sulphur and other bioactive compounds. Matthee et al. (2023) reported that freshwater fish species are usually more efficient in the uptake of drugs from the water compared to their seawater counterpart but will also depend on other factors such as chemical characteristics. This circumstance is because freshwater species absorb more water via their skin. On the other hand, something beneficial or harmless for one species could be detrimental or toxic to another. Hence, conducting toxicity tests on various fish species will help to understand the range of responses as well as potential differences in tolerance and susceptibility.

## CONCLUSION

The recommended dose for immersion of garlic juice extract is 600 ppm or below. At concentrations of 700 ppm and above, fish started to die due to oxidised sulphur toxicity. However, the present finding has unveiled that garlic juice extract is safe to be used orally for hybrid grouper without

any toxicity effect. Therefore, the juice extract can be utilised as a prophylactic or treatment in aquaculture setup. The present data on acute toxicity will serve as a guideline for defining the recommended dosage of garlic juice extract in marine fish health management, especially for grouper species. However, other toxicity tests should be conducted in the freshwater fish species due to differences in biological behaviour, especially water and drug uptake levels via the skin.

## ACKNOWLEDGEMENTS

This work was supported by the Department of Fisheries Malaysia Development Fund (P21225010390001).

## REFERENCES

Agency for Toxic Substances and Disease Registry. (2023a). *Medical management guidelines for sulfur dioxide*. https://wwwn.cdc.gov/TSP/MMG/MMGDetails.aspx?mmgid=249&toxid=46

Agency for Toxic Substances and Disease Registry. (2023b). *ToxFAQs™ for sulfur trioxide and sulfuric acid*. https://wwwn.cdc.gov/TSP/ToxFAQs/ToxFAQsDetails.aspx?faqid=255&toxid=47

Almeida, J. A., Barreto, R. E., Novelli, E. L. B., Castro, F. J., & Moron, S. E. (2009). Oxidative stress biomarkers and aggressive behavior in fish exposed to aquatic cadmium contamination. *Neotropical Ichthyology*, *7*(1), 103-108. https://doi.org/10.1590/s1679-62252009000100013

Arome, D., & Chinedu, E. (2013). The importance of toxicity testing. *Journal of Pharmaceutical and BioSciences*, *4*, 146-148.

Bayan, L., Koulivand, P. H., & Gorji, A. (2014). Garlic: A review of potential therapeutic effects. *Avicenna Journal of Phytomedicine*, *4*(1), 1–14.

Chen, C., Cai, J., Liu, S.-Q., Qiu, G.-L., Wu, X.-G., Zhang, W., Chen, C., Qi, W.-L., Wu, Y., & Liu, Z.-B. (2019). Comparative study on the composition of four different varieties of garlic. *PeerJ*, *7*, e6442. https://doi.org/10.7717/peerj.6442

Department of Fisheries. (2022). *Perangkaan perikanan tahunan 2022* [Annual fisheries statistics 2022]. https://www.dof.gov.my/sumber/perangkaan-perikanan-i

Erhirhie, E. O., Ihekwereme, C. P., & Ilodigwe, E. E. (2018). Advances in acute toxicity testing: Strengths, weaknesses and regulatory acceptance. *Interdisciplinary Toxicology*, *11*(1), 5-12. https://doi.org/10.2478/intox-2018-0001

Farid, A., Yousry, M., & Safwat, G. (2022). Garlic (*Allium sativum* Linnaeus) improved inflammation and reduced cryptosporidiosis burden in immunocompromised mice. *Journal of Ethnopharmacology*, *292*, 115174. https://doi.org/10.1016/j.jep.2022.115174

Foysal, M. J., Alam, M., Momtaz, F., Chaklader, M. R., Sidik, M. A. B., Cole, A., Fotedar, R., & Rahman, M. M. (2019). Dietary supplementation of garlic (*Allium sativum*) modulates gut microbiota and health status of tilapia (*Oreochromis niloticus*) against *Streptococcus iniae* infection. *Journal of Aquaculture Research*, *50*(8), 2107–2116. https://doi.org/10.1111/are.14088

Fridman, S., Sinai, T., & Zilberg, D. (2014). Efficacy of garlic based treatments against monogenean parasites infecting the guppy (*Poecilia reticulata* (Peters)). *Veterinary Parasitology*, *203*(1-2), 51-58. https://doi.org/10.1016/j.vetpar.2014.02.002

Guo, J. J., Kuo, C. M., Hong, J. W., Chou, R. L., Lee, Y. H., & Chen, T. I. (2015). The effects of garlic-supplemented diets on antibacterial activities against *Photobacterium damselae* subsp. *piscicida* and *Streptococcus iniae* and on growth in cobia, *Rachycentron canadum*. *Aquaculture*, *435*, 111-115. https://doi.org/10.1016/j.aquaculture.2014.09.029

Herrera, M., Mancera, J. M., & Costas, B. (2019). The use of dietary additives in fish stress mitigation: Comparative endocrine and physiological responses. *Frontiers in Endocrinology*, *10*, 447. https://doi.org/10.3389/fendo.2019.00447

Islam, M. A., Amin, S. M. N., Brown, C. L., Juraimi, A. S., Uddin, M. K., & Arshad, A. (2021). Determination of median lethal concentration ($LC_{50}$) for endosulfan, heptachlor and dieldrin pesticides to African catfish, *Clarias gariepinus* and their impact on its behavioural patterns and histopathological responses. *Toxics*, *9*(12), 340. https://doi.org/10.3390/toxics9120340

Koch, H. P., & Lawson, L. D. (1996). *Garlic: The science and therapeutic application of* Allium sativum *L. and related species* (2nd ed.). Williams & Wilkins.

Lee, D.-H., Ra, C.-S., Song, Y.-H., Sung, K.-I., & Kim, J.-D. (2012). Effects of dietary garlic extract on growth, feed utilization and whole body composition of juvenile sterlet sturgeon (*Acipenser ruthenus*). *Asian-Australasian Journal of Animal Science*, *25*(4), 577-583. https://doi.org/10.5713/ajas.2012.12012

Lee, J.-Y., & Gao, Y. (2012). Review of the application of garlic, *Allium sativum*, in aquaculture. *Journal of the World Aquaculture Society*, *43*(4), 447-458. https://doi.org/10.1111/j.1749-7345.2012.00581.x

Matthee, C., Brown, A. R., Lange, A., & Tyler, C. R. (2023). Factors determining the susceptibility of fish to effects of human pharmaceuticals. *Environmental Science and Technology*, *57*(24), 8845-8862. https://doi.org/10.1021/acs.est.2c09576

Mercy, A. A., & Gopalakannan, A. (2018). Antibacterial activity of fresh garlic juice against *Vibrio* sp. isolated from shrimp farm water: An *in vitro* study. *International Journal of Current Microbiology and Applied Sciences*, *7*(5), 485-489. https://doi.org/10.20546/ijcmas.2018.705.060

Mohamad, N., Roseli, F. A. M., Azmai, M. N. A., Saad, M. Z., Yasin, I. S. M., Zulkiply, N. A., & Nasruddin, N. S. (2019). Natural concurrent infection of *Vibrio harveyi* and *V. alginolyticus* in cultured hybrid groupers in Malaysia. *Journal of Aquatic Animal Health*, *31*(1), 88-96. https://doi.org/10.1002/aah.10055

Nasukha, A., Septory, R., & Muzaki, A. (2021). Analysis of nitrogen and phosphate in hybrid grouper media for better understanding of water management practices. In *IOP Conference Series: Earth and Environmental Science* (Vol. 718, No. 1, p. 012029). IOP Publishing. https://doi.org/10.1088/1755-1315/718/1/012029

Petropoulos, S. A., Fernandes, Â., Ntatsi, G., Petrotos, K., Barros, L., & Ferreira, I. C. F. R. (2018). Nutritional value, chemical characterization and bulb morphology of Greek garlic landraces. *Molecules*, *23*(2), 319. https://doi.org/10.3390/molecules23020319

Ridzuan, M. S. M., Abdullah, A., Ramly, R., Mansor, N. N., Ramli, N., & Firdaus-Nawi, M. (2022). Current status and advances of fish vaccines in Malaysia. *Veterinary World*, *15*(2), 465-482. https://doi.org/10.14202/vetworld.2022.465-482

Shasany, A. K., Ahirwar, O. P., Sushil, K., & Khanuja, S. P. S. (2000). RAPD analysis of phenotypic diversity in the Indian garlic collection. *Journal of Medicinal and Aromatic Plant Sciences*, *22*(1B), 586-592.

Strickland, J., Clippinger, A. J., Brown, J., Allen, D., Jacobs, A, Matheson, J., Lowit, A., Reinke, E. N., Johnson, M. S., Quinn Jr., M. J., Mattie, D., Fitzpatrick, S. C., Ahir, S., Kleinstreuer, N., & Casey, W. (2018). Status of acute systemic toxicity testing requirements and data uses by U.S. regulatory agencies. *Regulatory Toxicology and Pharmacology*, *94*, 183-196. https://doi.org/10.1016/j.yrtph.2018.01.022

Sutthinon, P., Thongprajukaew, K., Saekhow, S., & Ketmanee, R. (2014). Juvenile hybrid grouper (*Epinephelus coioides* × *E. lanceolatus*) are euryhaline and can grow in a wide range of salinities. *Aquaculture International*, *23*, 671-682. https://doi.org/10.1007/s10499-014-9845-8

Syngai, G. G., Dey, S., & Bharali, R. (2016). Evaluation of toxicity levels of the aqueous extract of *Allium sativum* and its effects on the behavior of juvenile common carp (*Cyprinus carpio* L., 1758). *Asian Journal of Pharmaceutical and Clinical Research*, *9*(3), 417-421.

Valenzuela-Gutiérrez, R., Lago-Lestón, A., Vargas-Albores, F., Cicala, F., & Martínez-Porchas, M. (2021). Exploring the garlic (*Allium sativum*) properties for fish aquaculture. *Fish Physiology and Biochemistry*, *47*, 1179–1198. https://doi.org/10.1007/s10695-021-00952-7

Wanapat, M., Khejornsart, P., Pakdee, P., & Wanapat, S. (2008). Effect of supplementation of garlic powder on rumen ecology and digestibility of nutrients in ruminants. *Journal of the Science of Food and Agriculture*, *88*(13), 2231-2237. https://doi.org/10.1002/jsfa.3333

Yunus, K., Jaafar, A. M., & John, A. (2019). Acute-lethal toxicity ($LC_{50}$) effect of *Terminalia catappa* Linn. leaves extract on *Oreochromis niloticus* (red Nile tilapia) juveniles under static toxicity exposure. *Oriental Journal of Chemistry*, *35*(1), 270-274. https://doi.org/10.13005/ojc/350132

Zhang, Y., Liu, X., Ruan, J., Zhuang, X., Zhang, X., & Li, Z. (2020). Phytochemicals of garlic: Promising candidates for cancer therapy. *Biomedicine and Pharmacotherapy*, *123*, 109730. https://doi.org/10.1016/j.biopha.2019.109730

Pertanika J. Trop. Agric. Sci. 47 (4): 1391 - 1403 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# The Effect of Biofertilizer Dose on Growth and Yield of Four Maize Varieties in Indonesia

**Ali Ikhwan[1]*, Yogga Adi Pratama[1], Erny Ishartati[1] and Faridlotul Hasanah[2]**

*[1]Department of Agrotechnology, Faculty of Agriculture and Animal Science, University of Muhammadiyah Malang, Jl. Raya Tlogomas No. 246 Malang, East Java 65145, Indonesia*
*[2]Alumni of Soil Science Study Program, Graduate School, IPB University, Jl. Raya Dramaga, Bogor, West Java 16680, Indonesia*

## ABSTRACT

Maize is one of the most demanding food crops, but its current production is not sufficient yet. However, demand needs are met through imports. Furthermore, maize is mostly cultivated on marginal land, affecting productivity. Biofertilizer application is an effort to increase maize yield by improving soil quality. This study determines the effect of biofertilizer application in liquid and granule form with several doses on the growth and yield of four local maize varieties of Indonesia. The study was carried out using nested randomized complete block design with two factors, i.e., 7 biofertilizers (two formulas, i.e., liquid and granule with 3 levels of dosage) and 4 maize varieties. The field experiment was conducted in Tawangrenjeni, Turen, Malang, East Java. The results showed that the application of biofertilizers affects the growth and yield of several maize varieties. Granular biofertilizer at a dose of 150 g/plant showed the best growth and yield observed variables, and Bisi 99 showed the best performance compared with other maize varieties.

*Keywords*: Corn, plant growth-promoting rhizobacteria, productivity, rhizobacteria consortia

ARTICLE INFO

*Article history:*
Received: 22 November 2023
Accepted: 27 February 2024
Published: 29 November 2024

DOI: https://doi.org/10.47836/pjtas.47.4.20

*E-mail addresses*:
aliikhwan64@gmail.com (Ali Ikhwan)
pratama.ya@gmail.com (Yogga Adi Pratama)
ishartati.erny@gmail.com (Erny Ishartati)
faridlotulhasanah@gmail.com (Faridlotul Hasanah)
* Corresponding author

## INTRODUCTION

Maize (*Zea mays* L.) is one of the food crops in great demand in Indonesia. However, its current production is insufficient to meet demand, so imports often meet it. Furthermore, maize is mostly cultivated on marginal land with less fertility, which affects its productivity. Therefore, maize productivity in dry land needs to be increased

ISSN: 1511-3701
e-ISSN: 2231-8542

(Ikhwan, Iriany, et al., 2021). The application of biofertilizer is an effort to increase maize yield by improving soil quality. Microorganisms contained in biofertilizers have various capabilities ranging from the production of growth-enhancing substances to the release of substances that ameliorate the effects of various abiotic stress conditions such as drought, nutrient deficiencies, salinity, pH stress, and even pollutants (Odoh et al., 2020).

Previous research by Ikhwan, Septia, et al. (2021) has obtained and identified several rhizobacteria isolated from maize rhizosphere. The results of 16S rDNA sequencing compared with Genbank at National Center for Biotechnology Information (NCBI) showed that 10 isolates were phylogenetically close to several bacterial strains, such as *Raoultella terrigena*, *Serratia marcescens*, *Serratia nematodiphila*, *Enterobacter hormaechei*, *Enterobacter cancerogenous*, *Enterobacter cloacae*, *Enterobacter asburiae*, *Citrobacter murliniae*, and *Pseudomonas fluorescens*. These rhizobacteria genera are commonly used as inoculants for producing biofertilizers such as *Enterobacter*, *Pseudomonas*, and *Serratia* (Glick, 2021; Seenivasagan & Babalola, 2021).

In biofertilizer formulations, a combination of several rhizobacteria can be used because many bacteria can live together and establish mutualistic relationships (Olanrewaju & Babalola, 2019). Furthermore, not all rhizobacteria have the same mechanisms and roles in supporting plant growth and production so that more profits will be obtained (Malusà et al., 2016). Akhtar et al. (2018) reported that maize grows better on bacterial consortium application compared to single or double inoculation on *Fusarium*-infested soil. In addition, Irfan et al. (2019) also found similar benefits in saline environments. It may occur because the consortium changes many nutrients, increases microbial activity in the soil, and changes nutrients through the symbiotic association of bacteria and plant roots.

A combination of several rhizobacteria was applied to improve the growth and yield of maize. Efthimiadou et al. (2020) reported an increase in maize yield due to the application of *Azotobacter chroococcum*, *Bacillus subtilis*, *Bacillus megatherium*, and their mixes. Ikhwan, Iriany, et al. (2021) added that the bacteria consortium of four bacterial strains can improve maize yield. Moreover, Katsenios et al. (2022) also investigated the effectiveness of ten plant growth-promoting bacteria strains, applied separately, on sweet corn cultivation. However, they found that applying different bacteria strain treatments did not influence the yield and quality of sweet corn. Therefore, research on applying bacteria consortia with several capabilities and mechanisms to enhance the growth and yield of maize is needed. This research attempted to apply a biofertilizer composed of a combination of ten bacterial strains. This study investigated the effect of biofertilizer application in liquid and granular form on several doses on the growth and yield of four Indonesian maize varieties.

## MATERIALS AND METHODS

### Preparation of Biofertilizer

Biofertilizer composed of 10 bacterial candidates, i.e., *Raoultella terrigena*, *Serratia marcescens*, *Serratia nematodiphila*, *Enterobacter hormaechei*, *Enterobacter hormaeghel*, *Enterobacter cancerogenous*, *Enterobacter cloaceae*, *Citrobacter murliane*, and *Psedomonas fluorences*. Ikhwan, Septia, et al. (2021) isolated and purified these bacterial candidates. Before use, all isolates were mixed, grown in a medium of 2 g/L GrowMore 32-10-10 (USA), 2 g/L GrowMore 10-55-10 (USA), and 20% (v/v) molasses, and harvested in the fermenter (capacity of 2 L) as described by Ikhwan, Septia, et al. (2021).

Biofertilizer was produced using a method described by Ikhwan, Septia, et al. (2021) with some modifications. Production of liquid biofertilizer was carried out using a production fermenter (capacity of 500 L), with a media composition of 200 L of sterile distilled water by adding 2 g/L of red Grow More 32-10-10 (USA), 2 g/L of Grow More 10-55-10 (USA), and 10% (v/v) molasses. Granular biofertilizer was produced using a granulator (diameter of 2.5 m) with a composition of 20 kg of rice husk charcoal. The granulation process involves incorporating bacteria with a density of 1 x $10^9$, which is sprayed into the granulation machine using a sprayer. Rice husk charcoal was poured into the granulation machine, and then the bacterial starter was sprayed slowly until the raw material turned into granules. It took around 10 min, and then the granules were air-dried. The characteristics of the granules are perfectly round with quite hard density and a diameter of about 0.5-1.0 cm.

### Experimental Design and Data Analysis

The field experiment was conducted in Tawangrenjeni Village, Turen District, Malang Regency, East Java, Indonesia. This research was carried out using a nested randomized complete block design (RCBD) using two factors, where the main factor was the maize variety (V), and the nested factor was the dose of granule and liquid biofertilizer (P). The main factors were maize varieties consisting of 4 varieties, namely Bisi 18 (V1), Bisi 99 (V2), Pertiwi 6 (V3), and Pertiwi 3 (V4). The nesting factor was the dose of granule and liquid fertilizer (P), consisting of 7 levels, i.e., without treatment or control (P0), 50 g/plant of granular biofertilizer (P1), 100 g/plant of granular biofertilizer (P2), 150 g/plant of granular biofertilizer (P3), 50 ml/plant of liquid biofertilizer (P4), 100 ml/plant of liquid biofertilizer (P5), and 150 ml/plant of liquid biofertilizer (P6). A population of 18 plants for each treatment combination and 10 observation samples were taken randomly. Variables were observed and measured as described by Ikhwan, Iriany, et al. (2021), including plant height (cm), number of leaves, stem diameter (mm), ear weight (g), cobs weight (g), cobs dry weight (g), cobs diameter (mm), tip filling (%), and 100-grain weight (g). The data were analyzed using analysis of variance (ANOVA) to understand the effect of the treatments and then using Tukey's honestly significant difference (HSD) α 5% to find the best treatment.

## RESULTS AND DISCUSSION

Different biofertilizer doses influenced the growth and yield of several maize varieties. The treatments significantly affected plant height, number of leaves, stem diameter, ear weight, and 100-grain weight. The influence of the treatments on maize plant height and number of leaves started to appear 21 days after planting (DAP) (Tables 1 and 2). At the end of observation, the highest average plant height and leave number were shown by Bisi 99 with an application of 100 g/plant granular biofertilizer, and the lowest average value was in the treatment of Bisi 18 without biofertilizer application. The effect of biofertilizer application on several maize varieties did not appear on stem diameter at first and at the end of observation. However, it significantly affected stem diameter from 21 to 35 DAP (Table 3).

The application of biofertilizer, both in the form of liquid and granule, consistently produced better maize growth than the control. Based on observations of plant height, number of leaves, and stem diameter, the results tend to be better with granule and liquid biofertilizers compared to those without biofertilizers. The 100 g/plant granule biofertilizer treatment showed the highest plant height at the middle (21 DAP) and end (49 DAP) of observation. In contrast, Bisi 99 showed the highest plant height among other varieties at 21, 35, 42, and 49 DAP (Table 1). The difference in the growth between maize varieties is due to the specific characteristics of each variety and its response to the application of rhizobacteria formulation. However, the quantitative results of the HSD test in Tables 1 and 2 show that the application of biofertilizer significantly increased plant height and number of maize leaves compared to the control. These results follow the previous research reported by Onyia et al. (2020), which showed a significant improvement in the growth of maize due to biofertilizer application compared with the control.

*Pseudomonas fluences* affect maize growth and could increase plant height. They can also increase the agronomic variables of maize and plant root length (Bhattacharyya & Jha, 2012). *Pseudomonas aeruginosa* has a growth-promoting effect on *Zea mays* L, which grows in 1% benzene and 0.5% phenanthrene and can also increase agronomic variables. It could act as a biological fertilizer in soil contaminated with aromatic hydrocarbons (Wong-Villarreal et al., 2019).

The application of biofertilizer also affected the number of maize leaves variable. Based on the results obtained in Table 2, the treatment of liquid biofertilizer at a dose of 150 ml/plant showed the highest number of leaves at 21, 28, and 35 DAP. The increase in maize leaves could be affected by the presence of *Serrata marcescens*. Adoko (2021) reported that a combination of bacterial inoculum of S. *marcescens* and peat soil produces the best results of leaf area in maize (*Zea mays* L.). Halotolerant plant growth-promoting rhizobacteria (PGPR) can be able to reduce environmental or abiotic stress. *Enterobacter asburiane* can increase growth and yield under salinity conditions.

Table 1
*Plant height of four maize varieties under several biofertilizer treatments at 14 to 49 days after planting*

| Treatments | Average plant height, cm (days after planting) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 14 | | 21 | | 28 | | 35 | | 42 | | 49 | |
| V1P0 | 18.33 | a | 35.22 | a | 60.11 | a | 83.78 | a | 98.78 | a | 100.44 | a |
| V1P1 | 22.56 | a | 39.56 | abcd | 66.56 | abcd | 88.89 | abcd | 107.33 | abc | 122.33 | bcdefg |
| V1P2 | 23.33 | a | 38.00 | abc | 64.67 | abc | 86.00 | a | 102.89 | ab | 107.44 | ab |
| V1P3 | 24.11 | a | 41.89 | cdefghi | 74.67 | fgh | 99.44 | defg | 109.33 | bcd | 123.89 | cdefgh |
| V1P4 | 28.33 | a | 42.11 | cdefghi | 65.78 | abc | 85.33 | a | 108.78 | abc | 128.00 | cdefghij |
| V1P5 | 25.33 | a | 38.22 | abc | 67.56 | bcde | 87.67 | ab | 102.11 | ab | 116.56 | abcde |
| V1P6 | 24.56 | a | 46.78 | ijkl | 67.11 | bcde | 88.56 | abc | 110.44 | bcde | 114.22 | abcd |
| V2P0 | 20.00 | a | 35.67 | ab | 61.56 | ab | 87.67 | ab | 102.22 | ab | 119.78 | bcdef |
| V2P1 | 22.56 | a | 44.56 | efghij | 78.22 | ghi | 98.22 | fgh | 139.44 | l | 173.44 | n |
| V2P2 | 21.67 | a | 44.22 | defghij | 73.56 | efgh | 93.33 | cdef | 135.89 | kl | 175.44 | n |
| V2P3 | 24.22 | a | 49.78 | kl | 75.89 | fgh | 95.44 | efg | 133.11 | jkl | 169.11 | n |
| V2P4 | 19.78 | a | 41.22 | cdefg | 76.33 | fghi | 91.67 | bcde | 119.33 | defgh | 151.11 | lm |
| V2P5 | 22.78 | a | 46.11 | ghijkl | 67.56 | bcde | 105.44 | ij | 133.56 | jkl | 161.56 | mn |
| V2P6 | 21.44 | a | 41.00 | cdef | 69.78 | cdef | 87.67 | ab | 109.33 | bcd | 127.00 | cdefghi |
| V3P0 | 21.78 | a | 40.22 | bcde | 67.89 | bcde | 99.56 | gh | 107.67 | abc | 119.56 | bcdef |
| V3P1 | 22.33 | a | 48.22 | jkl | 78.78 | ghi | 112.22 | l | 128.78 | hijk | 144.67 | kl |
| V3P2 | 23.00 | a | 49.89 | l | 79.44 | hi | 112.00 | l | 126.44 | hijk | 144.22 | jkl |
| V3P3 | 22.67 | a | 47.56 | jkl | 78.33 | ghi | 112.44 | l | 125.33 | ghij | 142.78 | Ijkl |
| V3P4 | 22.89 | a | 46.22 | hijkl | 79.11 | ghi | 103.67 | hi | 115.78 | cdefg | 130.11 | defghijk |
| V3P5 | 23.00 | a | 48.11 | jkl | 78.44 | ghi | 111.11 | kl | 122.67 | fghi | 135.78 | fghijkl |
| V3P6 | 23.67 | a | 49.67 | kl | 77.44 | ghi | 110.00 | jkl | 120.44 | efgh | 131.22 | efghijk |
| V4P0 | 21.78 | a | 35.22 | a | 63.33 | abc | 87.56 | ab | 104.89 | ab | 113.11 | abc |
| V4P1 | 22.67 | a | 38.89 | abc | 67.00 | bcde | 95.33 | efg | 119.56 | efgh | 141.44 | Ijkl |
| V4P2 | 25.22 | a | 41.67 | cdefgh | 72.56 | defg | 95.33 | efg | 116.00 | cdefg | 138.78 | hijkl |
| V4P3 | 23.11 | a | 44.11 | defghij | 75.67 | fgh | 94.44 | gh | 115.11 | cdef | 137.78 | ghijkl |
| V4P4 | 24.56 | a | 47.11 | jkl | 82.89 | i | 105.67 | ijK | 127.67 | hijk | 147.89 | lm |
| V4P5 | 25.00 | a | 44.56 | efghij | 77.67 | ghi | 101.89 | hi | 131.67 | ijkl | 144.00 | jkl |
| V4P6 | 23.33 | a | 45.22 | fghijk | 80.11 | hi | 102.11 | hi | 124.56 | fghij | 149.67 | lm |
| CV (%) | 9.66 | | 9.29 | | 7.91 | | 4.99 | | 7.39 | | 10.32 | |

*Note*. Means followed by the same letter in the same column are not significantly different based on the honestly significant difference test at the α 5% level; CV = Coefficient of variation; V1 = Bisi 18; V2 = Bisi 99; V3 = Pertiwi 6; V4 = Pertiwi 3; P0 = Control; P1 = 50 g/plant of granular biofertilizer; P2 = 100 g/plant of granular biofertilizer; P3 = 150 g/plant of granular biofertilizer; P4 = 50 ml/plant of liquid biofertilizer; P5 = 100 ml/plant of liquid biofertilizer; P6 = 150 ml/plant of liquid biofertilizer

Table 2
*Number of maize leaves under several biofertilizer treatments on four maize varieties at 14 until 49 days after planting*

| Treatments | Average number of maize leaves (days after planting) | | | | | |
|---|---|---|---|---|---|---|
| | 14 | 21 | 28 | 35 | 42 | 49 |
| V1P0 | 5.33 a | 5.11 a | 5.33 a | 5.44 a | 5.44 a | 6.89 ab |
| V1P1 | 5.11 a | 6.11 ef | 6.11 bcd | 6.55 cde | 7.22 cdefgH | 7.56 cd |
| V1P2 | 5.11 a | 5.89 cdef | 6.11 bcd | 6.22 bcd | 6.78 bcd | 7.33 bc |
| V1P3 | 5.00 a | 6.11 ef | 6.22 bcde | 7.11 fg | 7.22 cdefgh | 6.89 ab |
| V1P4 | 5.00 a | 5.89 cdef | 6.56 efg | 6.78 ef | 7.00 cdef | 8.00 defg |
| V1P5 | 5.00 a | 5.67 bcd | 6.11 bcd | 6.56 cde | 7.11 cdefg | 7.89 cdef |
| V1P6 | 5.00 a | 5.89 cdef | 6.33 cde | 6.78 h | 7.89 hijk | 7.78 cde |
| V2P0 | 5.00 a | 5.44 ab | 5.89 b | 6.45 bcde | 6.56 bc | 7.56 cd |
| V2P1 | 4.89 a | 6.00 def | 6.44 defg | 7.56 gh | 8.89 l | 8.56 ghi |
| V2P2 | 4.89 a | 5.89 cdef | 6.67 fg | 7.78 ef | 8.44 kl | 8.89 i |
| V2P3 | 4.89 a | 5.89 cdef | 6.67 fg | 7.56 gh | 8.33 jkl | 8.56 ghi |
| V2P4 | 4.89 a | 5.78 bcde | 6.44 defg | 7.44 gh | 7.44 defgh | 8.22 efgh |
| V2P5 | 4.89 a | 5.78 bcde | 6.22 bcde | 7.44 gh | 8.22 ijkl | 8.89 i |
| V2P6 | 4.89 a | 5.67 bcd | 6.22 bcde | 6.67 def | 6.89 cde | 7.89 cdef |
| V3P0 | 4.89 a | 5.67 bcd | 5.89 b | 6.11 bc | 7.00 cdef | 7.44 bcd |
| V3P1 | 4.89 a | 6.22 fg | 6.44 defg | 6.56 cde | 7.78 ghijk | 8.67 hi |
| V3P2 | 4.78 a | 5.89 cdef | 6.00 bc | 6.11 bc | 7.67 fghij | 8.56 ghi |
| V3P3 | 4.78 a | 6.22 fg | 6.33 def | 6.56 cde | 7.78 ghijk | 8.89 i |
| V3P4 | 4.78 a | 6.00 def | 6.11 bcd | 6.11 bc | 7.56 efghi | 8.44 fghi |
| V3P5 | 4.78 a | 6.11 ef | 6.22 bcde | 6.44 bcde | 7.56 efghi | 8.44 fghi |
| V3P6 | 4.67 a | 6.56 g | 6.78 g | 6.78 ef | 7.33 defgh | 8.67 hi |
| V4P0 | 4.67 a | 5.11 a | 5.33 a | 6.00 b | 6.11 ab | 6.33 a |
| V4P1 | 4.56 a | 5.11 a | 6.33 def | 7.33 gh | 7.44 defgh | 8.22 efgh |
| V4P2 | 4.56 a | 5.56 bc | 6.00 bc | 6.22 bcd | 7.33 defgh | 7.89 cdef |
| V4P3 | 4.44 a | 5.56 bc | 6.22 bcde | 6.33 bcde | 7.33 defgh | 8.33 efghi |
| V4P4 | 4.33 a | 5.89 cdef | 6.67 fg | 7.11 fg | 7.78 hijk | 8.33 efghi |
| V4P5 | 4.33 a | 6.11 ef | 6.78 g | 7.44 gh | 7.44 defgh | 8.44 fghi |
| V4P6 | 4.00 a | 6.00 def | 6.67 fg | 7.33 gh | 7.89 hijk | 8.44 fghi |
| CV (%) | 6.20 | 5.13 | 5.18 | 6.90 | 8.83 | 7.00 |

*Note*. Means followed by the same letter in the same column are not significantly different based on the honestly significant difference test at the α 5% level; CV = Coefficient of variation; V1 = Bisi 18; V2 = Bisi 99; V3 = Pertiwi 6; V4 = Pertiwi 3; P0 = Control; P1 = 50 g/plant of granular biofertilizer; P2 = 100 g/plant of granular biofertilizer; P3 = 150 g/plant of granular biofertilizer; P4 = 50 ml/plant of liquid biofertilizer; P5 = 100 ml/plant of liquid biofertilizer; P6 = 150 ml/plant of liquid biofertilizer

Table 3
*Stem diameter of four maize varieties under several biofertilizer treatments at 14 until 49 days after planting*

| Treatments | Average stem diameter, mm (days after planting) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 14 | | 21 | | 28 | | 35 | | 42 | | 49 | |
| V1P0 | 4.06 | a | 6.37 | a | 11.49 | a | 13.54 | a | 13.72 | a | 14.84 | a |
| V1P1 | 6.19 | a | 8.29 | bc | 13.43 | bc | 16.63 | defgh | 17.40 | cdefg | 17.99 | a |
| V1P2 | 5.78 | a | 7.96 | b | 12.37 | ab | 14.94 | ab | 16.73 | bcd | 17.01 | a |
| V1P3 | 6.09 | a | 9.09 | bcde | 14.19 | cde | 16.29 | bcdef | 17.76 | defg | 18.07 | a |
| V1P4 | 5.33 | a | 9.04 | bcde | 13.67 | bcd | 16.01 | bcde | 16.88 | bcd | 17.49 | a |
| V1P5 | 4.49 | a | 9.17 | cde | 13.49 | bcd | 16.41 | cdefg | 16.96 | bcde | 17.31 | a |
| V1P6 | 5.11 | a | 9.68 | defg | 14.36 | cde | 16.23 | bcdef | 17.29 | bcdef | 17.11 | a |
| V2P0 | 5.02 | a | 8.10 | bc | 13.79 | bcd | 15.52 | bcd | 16.00 | bc | 18.07 | a |
| V2P1 | 5.92 | a | 8.93 | bcd | 14.66 | cdef | 18.40 | ijkl | 19.00 | ghi | 19.76 | a |
| V2P2 | 5.44 | a | 9.68 | defg | 15.42 | efg | 19.14 | klm | 19.89 | ijk | 21.31 | a |
| V2P3 | 6.58 | a | 10.34 | fghi | 13.67 | bcd | 17.72 | hij | 18.62 | fghi | 20.74 | a |
| V2P4 | 5.03 | a | 9.01 | bcde | 17.26 | hi | 18.32 | ijkl | 19.52 | hij | 21.39 | a |
| V2P5 | 5.79 | a | 9.74 | defg | 14.76 | cdefg | 18.99 | jklm | 21.09 | jkl | 21.19 | a |
| V2P6 | 6.07 | a | 8.91 | bcd | 14.87 | defg | 16.62 | defgh | 17.74 | defg | 18.57 | a |
| V3P0 | 5.42 | a | 8.92 | bcd | 15.57 | efg | 17.47 | fghi | 18.83 | fghi | 19.66 | a |
| V3P1 | 6.56 | a | 10.76 | ghi | 18.92 | j | 20.80 | no | 21.16 | kl | 21.96 | a |
| V3P2 | 6.88 | a | 11.97 | j | 18.84 | j | 20.89 | no | 21.22 | kl | 21.52 | a |
| V3P3 | 6.82 | a | 10.94 | hij | 18.91 | j | 21.39 | o | 21.66 | l | 22.79 | a |
| V3P4 | 6.22 | a | 10.71 | ghi | 18.78 | j | 20.62 | no | 20.97 | jkl | 21.37 | a |
| V3P5 | 6.40 | a | 10.96 | hij | 18.67 | ij | 19.51 | lmn | 20.89 | jkl | 21.71 | a |
| V3P6 | 6.34 | a | 11.42 | ij | 18.74 | j | 20.26 | mno | 21.90 | l | 22.66 | a |
| V4P0 | 4.32 | a | 5.90 | a | 11.54 | a | 15.11 | bc | 15.68 | ab | 17.06 | a |
| V4P1 | 5.02 | a | 9.21 | cdef | 14.66 | cdef | 17.83 | hijk | 18.27 | defgh | 18.56 | a |
| V4P2 | 5.78 | a | 9.17 | cde | 14.82 | cdefg | 15.31 | bcd | 15.97 | bc | 17.62 | a |
| V4P3 | 5.96 | a | 10.14 | efgh | 15.29 | efg | 15.69 | bcd | 17.29 | bcdef | 17.77 | a |
| V4P4 | 6.19 | a | 10.38 | ghi | 12.54 | ab | 17.26 | fghi | 18.33 | defghi | 18.04 | a |
| V4P5 | 5.78 | a | 10.47 | ghi | 16.06 | fgh | 17.16 | fghi | 18.51 | efghi | 18.87 | a |
| V4P6 | 6.09 | a | 9.64 | defg | 16.17 | gh | 18.06 | ijk | 18.22 | defgh | 18.61 | a |
| CV (%) | 12.97 | | 10.37 | | 8.11 | | 6.97 | | 7.57 | | 8.73 | |

*Note*. Means followed by the same letter in the same column are not significantly different based on the honestly significant difference test at the α 5% level; CV = Coefficient of variation; V1 = Bisi 18; V2 = Bisi 99; V3 = Pertiwi 6; V4 = Pertiwi 3; P0 = Control; P1 = 50 g/plant of granular biofertilizer; P2 = 100 g/plant of granular biofertilizer; P3 = 150 g/plant of granular biofertilizer; P4 = 50 ml/plant of liquid biofertilizer; P5 = 100 ml/plant of liquid biofertilizer; P6 = 150 ml/plant of liquid biofertilizer

The influence of biofertilizer application on several maize varieties was seen in cobs dry weight, cobs diameter, 100-grain weight, and tip filling (Table 4). Biofertilizer application also affected the ear weight and cob weight of several maize varieties (Figure 1). Bisi 99 showed the highest ear weight, cobs weight, and cobs dry weight, while Pertiwi 3 showed the highest 100-grain weight, cobs diameter, and tip filling, among other varieties. The dose between the two forms of biofertilizer similarly showed good performance on maize yield variables.

Maize (*Zea mays* L.) is a cereal crop that can be grown in various climatic conditions. Rhizobacteria isolated from holophytes were found to increase the vegetative growth parameters of maize under induced salinity (Aslam & Ali, 2018). Besides, rhizobacteria can increase plant growth in an area susceptible to nematodes. *Pseudomonas fluences* can significantly increase the growth of bitter melon plants that have been given the nematode *Renifom resinormis* (Humphries et al., 2021). Furthermore, Ali et al. (2022) reported that inoculation of *Enterobacter cloceae* could suppress the abiotic stress of maize and promote yield, fresh weight, dry weight, and leaf area of maize.

Apart from the ability to reduce the impact of biotic and abiotic stress, bacterial consortia in this study have also been proven to promote the growth and yield of maize. Kämpfer et al. (2016) stated that *Enterobacter* sp. can play an important role in improving plant growth. Some *Enterobacter* strains can play an important role in plant-microbial interactions in the biocontrol mechanism, wherein the results of the study showed that the treatment of granule biological fertilizers at a dose of 150 g/plant had the highest values in the parameters of dry weight and 100-grain weight. Mehta et al. (2015) also stated that the maize yield increases with the inoculation of *Pseudomonas fluences*. *Pseudomonas* sp. can also increase fruit length, root weight, and root length. *P. flurences* bacteria also have many roles in phosphate solubilization and the production of auxin and gibberellins. *Enterobacter* bacteria can act as a plant growth promoting (PGP) in rice and some maize strains (Toribio-Jiménez et al., 2017). Moreover, Devi et al. (2016) also reported that *S. marcescens* strain AL2-16 can produce indole acetic acid in a medium supplemented with l-tryptophan, solubilized inorganic phosphate, and gave positive results for ammonia production. Sutio et al. (2023) also highlighted the role of *S. marcescens* strain NPKC3_2_21 as P-solubilizing bacteria that enhance the availability of P by producing organic acids and entomopathogenic bacteria to insects, especially *Spodoptera litura*.

In summary, the application of biofertilizer significantly increased maize growth and yield compared to the control. The results confirmed that biofertilizers could contribute as a new cultivation practice for sustainable growth and productivity of grain crops. The rhizobacteria consortium had a positive impact on growth and yield variables; no antagonistic reactions were seen. The difference in the growth

Table 4
*Yield observation of four maize varieties under several biofertilizer treatments*

| | Treatments | Cobs dry weight (g) | | 100-grain weight (g) | | Cobs diameter (mm) | | Tip filling (%) |
|---|---|---|---|---|---|---|---|---|
| Bisi 18 | Control (P0) | 116.78 | a | 32.83 | a | 42.93 | a | 95.00 c |
| | Granule 50 g (P1) | 170.00 | cdef | 41.54 | fg | 48.76 | efghi | 96.89 cde |
| | Granule 100 g (P2) | 163.67 | cde | 39.02 | def | 46.18 | bcde | 97.11 cde |
| | Granule 150 g (P3) | 168.44 | cdef | 41.71 | fg | 46.07 | bcde | 97.22 cde |
| | Liquid 50 ml (P4) | 156.44 | bcd | 38.26 | def | 45.59 | abcd | 97.56 de |
| | Liquid 100 ml (P5) | 166.44 | cdef | 37.28 | bcde | 46.51 | cdef | 97.56 de |
| | Liquid 150 ml (P6) | 135.56 | ab | 37.77 | cdef | 44.48 | abc | 95.78 cd |
| Bisi 99 | Control (P0) | 134.67 | ab | 33.50 | ab | 43.61 | ab | 92.11 b |
| | Granule 50 g (P1) | 232.56 | jkl | 39.88 | def | 49.34 | ghij | 96.00 cd |
| | Granule 100 g (P2) | 240.00 | kl | 35.93 | abcd | 49.37 | ghij | 97.22 cde |
| | Granule 150 g (P3) | 227.56 | jkl | 36.16 | abcde | 49.03 | fghi | 97.22 cde |
| | Liquid 50 ml (P4) | 248.45 | l | 37.12 | bcde | 50.06 | hij | 97.78 de |
| | Liquid 100 ml (P5) | 206.89 | ghij | 33.85 | abc | 48.03 | defghi | 97.00 cde |
| | Liquid 150 ml (P6) | 210.44 | hij | 33.41 | ab | 47.34 | defgh | 96.89 cde |
| Pertiwi 6 | Control (P0) | 129.67 | ab | 40.32 | efg | 44.29 | abc | 87.22 a |
| | Granule 50 g (P1) | 187.56 | efghi | 49.91 | ij | 49.44 | ghij | 97.89 de |
| | Granule 100 g (P2) | 182.00 | cdefg | 46.69 | hi | 48.73 | efghi | 97.56 de |
| | Granule 150 g (P3) | 193.22 | fghi | 48.35 | hij | 48.53 | efghi | 97.33 cde |
| | Liquid 50 ml (P4) | 155.22 | bc | 44.44 | gh | 46.76 | cdefg | 97.78 de |
| | Liquid 100 ml (P5) | 155.78 | bc | 44.48 | gh | 47.06 | cdefg | 98.00 de |
| | Liquid 150 ml (P6) | 163.11 | cde | 49.19 | ij | 47.48 | defgh | 97.89 de |
| Pertiwi 3 | Control (P0) | 171.33 | cdef | 39.85 | def | 46.90 | cdefg | 92.33 b |
| | Granule 50 g (P1) | 213.56 | ijk | 54.74 | l | 54.30 | l | 97.89 de |
| | Granule 100 g (P2) | 191.56 | fghi | 54.52 | kl | 53.11 | kl | 97.89 de |
| | Granule 150 g (P3) | 167.56 | cdef | 48.55 | hij | 50.57 | ijk | 98.22 e |
| | Liquid 50 ml (P4) | 171.56 | cdef | 50.56 | ijkl | 53.32 | kl | 98.44 e |
| | Liquid 100 ml (P5) | 182.11 | cdefg | 50.75 | ijkl | 51.93 | jkl | 98.11 e |
| | Liquid 150 ml (P6) | 183.44 | defgh | 51.38 | jkl | 52.03 | jkl | 97.89 de |
| CV (%) | | 13.13 | | 8.51 | | 5.05 | | 2.18 |

*Note*. Means followed by the same letter in the same column are not significantly different based on the honestly significant difference test at the α 5% level; CV = Coefficient of variation; P0 = Control; P1 = 50 g/plant of granular biofertilizer; P2 = 100 g/plant of granular biofertilizer; P3 = 150 g/plant of granular biofertilizer; P4 = 50 ml/plant of liquid biofertilizer; P5 = 100 ml/plant of liquid biofertilizer; P6 = 150 ml/plant of liquid biofertilizer

between maize varieties is due to the specific characteristics of each variety and its response to the application of rhizobacteria formulation. Generally, granular biofertilizers showed better performance in improving plant growth and yield compared to liquid biofertilizers. The 100-grain weight increased up to 37% compared to the control. The use of granular biological fertilizers provides practical implications and advantages for fertilization and distribution. Furthermore, the urge for more sustainable cultivation practices has led researchers worldwide to investigate the ability of rhizobacteria to enhance plant growth and yield. Different parameters should be examined, and the application procedure should be optimized to understand the activity of rhizobacteria on crop productivity and provide practical recommendations for supporting the agricultural field.

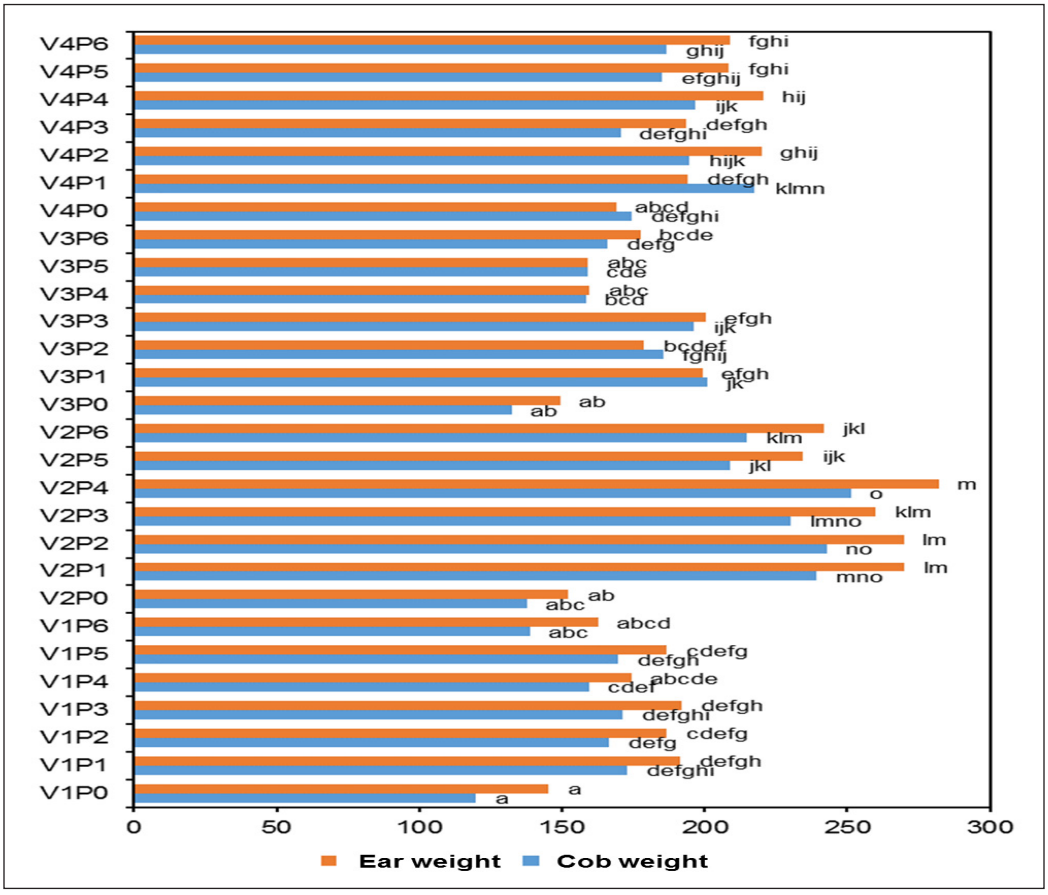



*Figure 1*. Ear weight and cob weight of four maize varieties under several biofertilizer treatments
*Note*. The bar charts followed by the same letter in the same color are not significantly different based on the honestly significant difference test at the α 5% level; V1 = Bisi 18; V2 = Bisi 99; V3 = Pertiwi 6; V4 = Pertiwi 3; P0 = Control; P1 = 50 g/plant of granular biofertilizer; P2 = 100 g/plant of granular biofertilizer; P3 = 150 g/plant of granular biofertilizer; P4 = 50 ml/plant of liquid biofertilizer; P5 = 100 ml/plant of liquid biofertilizer; P6 = 150 ml/plant of liquid biofertilizer

## CONCLUSION

Maize varieties and biofertilizer doses significantly influenced the growth and yield of maize. The form and dose of fertilizer affected the growth and yield variables significantly, with the granule form at a dose of 150 g/plant showing the best growth (plant height, plant height, number of leaves, and stem diameter) and yield (ear weight and 100-grain weight) compared to control. Bisi 99 showed the best performance in growth and yield variables compared to other maize varieties. Further research is needed regarding phytohormone bioassays, changes in soil nutrient status, and efficacy against insects due to the application of biofertilizers. Moreover, the potential of rhizobacteria as a biofertilizer needs to be explored.

## ACKNOWLEDGEMENTS

This research was funded by the National Research and Innovation Agency (BRIN), Deputy for National Research and Innovation Facilitation (Grant Number: 65/II.7/HK/2022). The authors also gratefully acknowledge the Research Team and Department of Agrotechnology, Faculty of Agriculture and Animal Science, University of Muhammadiyah Malang, for supporting this research.

## REFERENCES

Adoko, M. Y., Sina, H., Amogou, O., Agbodjato, N. A., Noumavo, P. A., Aguégué, R. M., Assogba, S. A., Adjovi, N. A., Dagbénonbakin, G., Adjanohoun, A., & Baba-Moussa, L. (2021). Potential of biostimulants based on PGPR rhizobacteria native to Benin's soils on the growth and yield of maize (*Zea mays* L.) under greenhouse conditions. *Open Journal of Soil Science*, *11*(3), 177-196. https://doi.org/10.4236/ojss.2021.113010

Akhtar, N., Naveed, M., Khalid, M., Ahmad, N., Rizwan, M., & Siddique, S. (2018). Effect of bacterial consortia on growth and yield of maize grown in *Fusarium* infested soil. *Soil and Environment*, *37*(1), 35-44. https://doi.org/10.25252/SE/18/872

Ali, B., Wang, X., Saleem, M. H., Sumaira., Hafeez, A., Afridi, M. S., Khan, S., Zaib-Un-Nisa., Ullah I., do Amaral Júnior A. T., Alatawi, A., & Ali, S. (2022). PGPR-mediated salt tolerance in maize by modulating plant physiology, antioxidant defense, compatible solutes accumulation and bio-surfactant producing genes. *Plants*, *11*(3), 345. https://doi.org/10.3390/plants11030345

Aslam, F., & Ali, B. (2018). Halotolerant bacterial diversity associated with *Suaeda fruticosa* (L.) Forssk. improved growth of maize under salinity stress. *Agronomy*, *8(8)*, 131. https://doi.org/10.3390/agronomy8080131

Bhattacharyya, P. N., & Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): Emergence in agriculture. *World Journal of Microbiology and Biotechnology*, *28*, 1327–1350. https://doi.org/10.1007/s11274-011-0979-9

Devi, K. A., Pandey, P., & Sharma, G. D. (2016). Plant growth-promoting endophyte *Serratia marcescens* AL2-16 enhances the growth of *Achyranthes aspera* L., a medicinal plant. *HAYATI Journal of Biosciences*, *23*(4), 173–180. https://doi.org/10.1016/j.hjb.2016.12.006

Efthimiadou, A., Katsenios, N., Chanioti, S., Giannoglou, M., Djordjevic, N., & Katsaros, G. (2020). Effect of foliar and soil application of plant growth promoting bacteria on growth, physiology, yield and seed quality of maize under

Mediterranean conditions. *Scientific Reports*, *10*, 21060. https://doi.org/10.1038/s41598-020-78034-6

Glick, B. R. (2012). Plant growth-promoting bacteria: Mechanisms and applications. *Scientifica*, *2012*, 963401. https://doi.org/10.6064/2012/963401

Humphries, R., Campeau, S., Davis, T. E., Nagaro, K. J., LaBombardi, V. J., Franklin, S., Heimbach, L., & Dwivedi, H. P. (2021). Multicenter evaluation of ceftazidime-avibactam susceptibility testing of *Enterobacterales* and *Pseudomonas aeruginosa* on the Vitek 2 system. *Journal of Clinical Microbiology*, *59*(3), e01870-20. https://doi.org/10.1128/jcm.01870-20

Ikhwan, A., Iriany, A., Ishartati, E., & Hasanah, F. (2021). Formulation of bacterial consortium for improvement growth and yield of maize (*Zea mays* L.). *SAINS TANAH - Journal of Soil Science and Agroclimatology, 18*(1), 89-97. http://doi.org/10.20961/stjssa.v18i1.46003

Ikhwan, A., Septia, E. D., & Novita, B. A. (2021). Molecular identification of potential rhizobacteria isolated from maize (*Zea mays* L.). In *IOP Conference Series: Earth and Environmental Science* (Vol. 985, No. 1, p. 012010). IOP Publishing. https://doi.org/10.1088/1755-1315/985/1/012010

Irfan, M., Zahir, Z. A., Asghar, H. N., Khan, M. Y., Ahmad, H. T., & Ali, Q. (2019). Effect of multi-strain bacterial inoculation with different carriers on growth and yield of maize under saline conditions. *International Journal of Agriculture and Biology*, *22*, 1407-1414. https://doi.org/10.17957/IJAB/15.1215

Kämpfer, P., McInroy, J. A., Doijad, S., Chakraborty, T., & Glaeser, S. P. (2016). *Kosakonia pseudosacchari* sp. nov., an endophyte of *Zea mays*. *Systematic and Applied Microbiology*, *39*(1), 1-7. https://doi.org/10.1016/j.syapm.2015.09.004

Katsenios, N., Andreou, V., Sparangis, P., Djordjevic, N., Giannoglou, M., Chanioti, S., Kasimatis, C.-N., Kakabouki, I., Leonidakis, D., Danalatos, N., Katsaros, G., & Efthimiadou, A. (2022). Assessment of plant growth promoting bacteria strains on growth, yield and quality of sweet corn. *Scientific Reports*, *12*, 11598. https://doi.org/10.1038/s41598-022-16044-2

Malusà, E., Pinzari, F., & Canfora, L. (2016). Efficacy of biofertilizers: Challenges to improve crop production. In D. P. Singh, H. B. Singh, & R. Prabha (Eds), *Microbial inoculants in sustainable agricultural productivity* (pp. 17-40). Springer. https://doi.org/10.1007/978-81-322-2644-4_2

Mehta, S. K., Baheti, B. L., Rathore, B. S., & Nama, C. P. (2015). Botanicals - An effective tool for the management of maize cyst nematode, *Heterodera zeae* on maize (*Zea mays* L.). *Journal of Biopesticides*, *8*(2), 62–67. http://doi.org/10.57182/jbiopestic.8.2.62-67

Odoh, C. K., Sam, K., Zabbey, N., Eze, C. N., Nwankwegu, A. S., Laku, C., & Dumpe, B. B. (2020). Microbial consortium as biofertilizers for crops growing under the extreme habitats. In A. N. Yadav, J. Singh, A. A. Rastegari, & N. Yadav (Eds.), *Plant microbiomes for sustainable agriculture: Sustainable development and biodiversity* (Vol. 25, pp. 381-424). Springer. https://doi.org/10.1007/978-3-030-38453-1_13

Olanrewaju, O. S., & Babalola, O. O. (2019). Bacterial consortium for improved maize (*Zea mays* L.) production. *Microorganisms*, *7*(11), 519. https://doi.org/10.3390/microorganisms7110519

Onyia, C. O., Okoh, A. M., & Irene, O. (2020). Production of plant growth-promoting bacteria biofertilizer from organic waste material and evaluation of its performance on the growth of corn (*Zea mays*). *American Journal of Plant Sciences*, *11*(2), 189-200. https://doi.org/10.4236/ajps.2020.112015

Seenivasagan, R., & Babalola, O. O. (2021). Utilization of microbial consortia as biofertilizers and biopesticides for the production of feasible agricultural product. *Biology*, *10*(11), 1111. https://doi.org/10.3390/biology10111111

Sutio, G., Afifah, A. N., Maharani, R., & Basri, M. (2023). *Serratia marcescens* strain NPKC3_2_21 as endophytic phosphate solubilizing bacteria and entomopathogen: Promising combination approach as rice biofertilizer and biopesticide. *Biodiversitas Journal of Biological Diversity*, *24*(2), 901-909.

Toribio-Jiménez, J., Rodriguez-Barrera, M., Hernandez-Florez, G., Ruvalcaba-Ledezma, J. C., Castellanos-Escazmilla, M., & Romero-Ramirez, Y. (2017). Isolation and screening of bacteria from *Zea mays* plant growth promoters. *Revista Internacional de Contaminacion Ambiental*, *33*, 143–150. http://doi.org/10.20937/RICA.2017.33.esp01.13

Wong-Villarreal, A., Yañez-Ocampo, G., Hernández-Nuñez, E., Corzo-González, H., Giácoman-Vallejos, G., González-Sánchez, A., Gómez-Velasco, D., González, S. I. R., López-Baez, O., & Espinosa-Zaragoza, S. (2019). Bacteria from *Jatropha curcas* rhizosphere, degrades aromatic hydrocarbons and promotes growth in *Zea mays*. *Open Agriculture*, *4*(1), 641-649. https://doi.org/10.1515/opag-2019-0066

Pertanika J. Trop. Agric. Sci. 47 (4): 1405 - 1428 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

*Review Article*

# Review of the Innovations and Challenges in Developing Rapid Colorimetry and Turbidity NPK Soil Test Kits for Commercial Soil Nutrient Analysis

**Melissa Mei Teng Lok[1], Ngai Paing Tan[1], Yei Kheng Tee[2] and Christopher Boon Sung Teh[1]***

*[1]Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
*[2]Cocoa Upstream Technology Department, Malaysian Cocoa Board, Sg. Dulang Road, P.O. Box 30, 36307 Sg. Sumun, Perak, Malaysia*

## ABSTRACT

This study reviews the progress and challenges in developing rapid colorimetric and turbidimetric test kits for commercial soil nutrient analyses, focusing on nitrogen, phosphorus, and potassium. Although common laboratory analytical techniques are accurate, they are often expensive and require advanced technical skills. Consequently, commercial *in situ* soil test kits have gained attention as potential alternatives to these tests. These kits use universal extractants that can simultaneously extract multiple nutrients and colorimetry and turbidimetry for nutrient determination. A critical aspect of these kits is the selection of suitable extractants that are effective under various soil conditions, chemically safe, compatible with analytical systems, and have a long shelf life. This review assesses the efficacy of several extractants in conjunction with colorimetric and turbidimetric reagents. Examples include H3A-4 and Kelowna extractants using the zinc–Griess reagent, salicylic method, molybdenum yellow method, and sodium tetraphenyl boron method for nitrate, ammonium, phosphorus, and potassium, respectively. These extractants and reagents have been highlighted for their adaptability, safety, rapid color formation, and minimal compatibility issues, making them promising candidates for rapid test kit development. However, rapid soil test kits are designed to provide a general understanding of soil nutrient status. They are not intended

ARTICLE INFO

*Article history:*
Received: 20 January 2024
Accepted:19 March 2024
Published: 29 November 2024

DOI: https://doi.org/10.47836/pjtas.47.4.21

*E-mail addresses*:
melissa.meiteng@gmail.com (Melissa Mei Teng Lok)
ngaipaing@upm.edu.my (Ngai Paing Tan)
tee_yei@koko.gov.my (Yei Kheng Tee)
chris@upm.edu.my (Christopher Boon Sung Teh)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

to replace detailed laboratory analyses in situations where precision is critical. Additionally, these kits should be validated against standard laboratory methods to determine their accuracy and reliability. This review highlights the importance of balancing practicality and accuracy when developing rapid soil test kits for soil nutrient analysis.

*Keywords*: Colorimetry, commercial soil nutrient rapid test kit, NPK, turbidity, universal extractant

## INTRODUCTION

Integrated soil fertility management has significantly improved crop yields, bolstered resistance to environmental stresses, and curbed greenhouse gas emissions (Roobroeck et al., 2016). However, farmers often face challenges due to uncertainty in determining the appropriate quantity and type of fertilizer required to maintain soil fertility. This uncertainty underscores the significance of conducting soil nutrient analyses before fertilization to ensure optimal crop yield and quality.

Conventional farming practices have increasingly led to the overapplication of chemical fertilizers, contributing to the global wastage of approximately 50–65% of N and P in agricultural fields (Ritchie, 2021). Such practices not only squander farmers' resources but also pose environmental hazards, including eutrophication, harmful effects on aquatic ecosystems, and adverse effects on human health. Conversely, insufficient nutrient levels can result in poor crop quality and yield, highlighting the need for appropriate fertilizer application for sustainable farming.

Despite offering comprehensive and highly precise data, laboratory soil nutrient analysis is typically not feasible for smallholder farmers because of high costs, lengthy time requirements, and technical complexity. This process involves handling hazardous chemicals, using specific extraction methods for each nutrient, and using expensive equipment such as inductively coupled plasma (ICP) mass spectrometry, atomic absorption spectroscopy (AAS), and auto analyzers. In Malaysia, the service cost per nutrient element is at least MYR 30, and results are obtained in days to months. These constraints made the analysis inaccessible for many smallholders, thereby impeding effective fertilization programs and crop production.

Commercial colorimetric soil nutrient test kits have emerged as viable alternatives in response to these challenges. These kits categorize soil nutrient concentrations into practical levels (low, medium, and high) and provide approximate readings visible to the naked eye. The affordability and capacity to yield immediate results, with costs ranging between MYR 15 and 40 in Malaysia, represent significant advancements. However, concerns arise because despite the availability of over 90 types of rapid soil test kits online, more than half lack validation by credible independent authorities (Dimkpa et al., 2017). Scientific assessments have shown that the accuracy of these kits varies from 33 to 94% compared with analytical

laboratory results (Faber et al., 2007). This variability raises questions about reliability. Nevertheless, when accuracy and precision are ensured, rapid test kits that provide approximate readings are still acceptable for an immediate understanding of soil nutrient status.

This review thoroughly examined the efficacy of rapid soil nutrient testing using colorimetric and turbidimetric systems, focusing on their accuracy, precision, and practicality in fertilization management.

## METHODOLOGY

The literature search used ScienceDirect (https://www.sciencedirect.com) and Google Scholar (https://scholar.google.com) with the following initial sets of keywords: "soil nutrient analysis", "NPK extraction", "universal extractant", "rapid soil test kits", "colorimetry", and "turbidimetry". Subsequently, the search was refined by incorporating specific keywords such as "Morgan", "Kelowna", "$SrCl_2$ universal extractant", and "H3A" to focus on universal extractants. For colorimetric and turbidimetric methods, the targeted keywords included "Nessler", "Berthelot–salicylic method", "Griess method", "$NO_3^-$ salicylic method", "molybdenum blue P method", "molybdenum yellow P method", "sodium tetraphenyl boron turbidimetry reagent", and "crown ether-modified gold particles". This approach identified scholarly articles discussing the simultaneous extraction of NPK in the context of colorimetric and turbidimetric methods using specific universal extractants and reagents.

Articles were selected based on their relevance to the study objectives and the timeline for publication. This approach traced the evolution of NPK assessment methodologies from pioneering studies to more recent advancements. The emphasis was placed on publications published after 2010 that were aligned with the latest developments in NPK soil nutrient analysis methodologies.

The initial search yielded 187 relevant papers. Of these, 75 were chosen because of their substantial contribution to the literature on rapid soil NPK nutrient analysis. The selection process prioritized studies that focused on universal soil extraction methods and colorimetry- and turbidimetry-based determination methods. These studies have significantly enriched the discussion of contemporary soil nutrient assessment methodologies, with a specific focus on NPK analysis. This rigorous selection process ensured the collation of pertinent literature for this review, thereby providing a comprehensive overview of the current state of soil nutrient analyses.

## RAPID SOIL NPK TEST KIT DEVELOPMENT

Bünemann et al. (2018) reported that the testing frequency for N, P, and K in soil was greater than that for other soil quality indicators. It is unsurprising given the significance of N, P, and K in plants.

$NO_3^-$ and $NH_4^+$ are key N sources in plant roots and influence plants' physiological and morphological aspects. The effects of phytohormones include

genome regulation, seed dormancy, floral induction, and phytohormone regulation (Hachiya & Sakakibara, 2016).

Soil P, often in its organic form, undergoes mineralization to orthophosphate (Pi), primarily as dihydrogen P ($H_2PO_4^-$) and hydrogen P ($HPO_4^{2-}$) ions. These ions diffuse into plant roots and are transported through symplastic pathways to the xylem and cytoplasm. Plants use P for several critical functions, including energy generation, nucleic acid synthesis, photosynthesis, respiration, metabolic regulation, and membrane synthesis and stability (Vance et al., 2003).

Vascular plants actively absorb K through specialized transporters and channels. This uptake is essential for various plant functions, including enzyme activation, membrane transport, anion neutralization, osmoregulation, photosynthesis facilitation, and assimilation product transport (Y. Wang & Wu, 2013). Therefore, assessing soil N, P, and K levels has become a priority in developing rapid test kits, given their crucial role as macronutrients essential for plant health.

Developing a rapid soil test kit typically begins with the simultaneous extraction of multiple targeted nutrients, such as N, P, and K, from soil samples using a single extractant, often referred to as a universal soil extractant. The concentrations of nutrients in the extractant supernatant were visually assessed using colorimetry and turbidimetry. The pioneering work of Morgan (1941), who introduced a colorimetric and turbidimetric diagnostic system using a universal extractant, represents a significant advancement in rapid laboratory testing protocols. This methodology has served as a foundational reference for the innovation and development of commercial rapid soil test kits, facilitating quicker and more accessible nutrient analysis.

## NPK Universal Soil Extractant

A universal soil extractant, or multiple-nutrient extractant, is a single solution that simultaneously extracts multiple nutrients from soil samples (Haney et al., 2010; Jones Jr., 1990). Numerous studies have compared their results with those obtained using specific standard extractants to evaluate the efficacy of such extractants. For instance, 2 N potassium chloride (KCl) is typically used to extract $NH_4^+$ and $NO_3^-$; Olsen, Bray, and Mehlich extractants are used for P, and 1 M ammonium acetate ($NH_4OAc$) and Mehlich-3 are used for K (De Silva et al., 2015; Sahrawat, 1979; Simard & Deschênes, 1992; Simard et al., 1991; van Lierop, 1986). This comparative approach is crucial for determining the effectiveness of a universal extractant for extracting various nutrients from soil, thereby validating its use in soil nutrient analysis.

### *Morgan Extractant*

The Morgan universal extractant, composed of 0.73 M sodium acetate ($C_2H_3NaO_2$) and 0.52 N acetic acid (HOAc), was introduced in 1941 as a pioneering solution capable of simultaneously extracting various elements from soil, including $NO_3^-$, $NH_4^+$, P, K, Ca, Mg, other micronutrients, and heavy

metals (Morgan, 1941). A validation study conducted in the 1970s demonstrated that the Morgan extractant achieved over 80% recovery of inorganic N in sandy clay loam soils from an old alluvium series (Sahrawat & Prasad, 1975). Further research comparing $NH_4^+$ extraction methods in tropical rice soils found that the Morgan extractant performed comparably with 2 N KCl in five of the seven tested soils (Sahrawat, 1979).

In 1982, Wolf modified the Morgan extractant by adding diethylenetriaminepentaacetic acid (DTPA) and performing a vigorous shaking step for 5 min (Jones Jr., 1990). Although the Morgan–Wolf extractant extracted less K than the other methods, it showed a moderately significant variation in P extraction; it extracted more P in high-pH soils, dissolving calcium–phosphate, but less P in low-pH soils, where it struggled to dissolve iron–phosphate and aluminum–phosphate (Masjkur, 2009). In 2015, a strong correlation ($r = 0.73$) was established between the Morgan reagent and the Olsen method, indicating the suitability of the Morgan reagent for rapid colorimetric P determination in mildly acidic to basic soils (De Silva et al., 2015). The original Morgan and modified Morgan–Wolf extractants have been recommended for use in acidic soils (Kumawat et al., 2017).

### *Kelowna Extractant*

The $NH_4OAc$ with ammonium fluoride ($NH_4F$) extractant, a modification of the Morgan and Bray extractants, was developed to enhance the extraction of multiple nutrients, particularly P and K, in acidic soils (McIntosh, 1969). Later, the Kelowna extractant, comprising 0.25 N HOAc and 0.015 N $NH_4F$, demonstrated a strong linear correlation ($r = 0.99$) with the 2 N KCl method when using steam distillation alongside Devarda alloy for $NO_3^-$ determination (van Lierop, 1986). The results also showed strong correlations with both the Bray-1 and Olsen methods ($r > 0.99$) for acidic and calcareous soils. It extracted approximately 2.5 times more P than the Olsen method. The Kelowna extractant was developed to improve the Bray solution, a recognized P extraction solution for acidic soils, by substituting 2 N hydrochloric acid (HCl) with 0.25 N HOAc (van Lierop, 1988). Additionally, the Kelowna extractant exhibited a strong linear correlation ($r = 0.99$) with 1 N $NH_4OAc$ to extract K from acidic and calcareous soils (van Lierop & Gough, 1989). In 1996, the Kelowna multiple-nutrient extractant was used as a reference for extracting $NO_3^-$, P, K, Ca, Mg, Na, and sulfur from agricultural soils in British Columbia (Gough, 1996).

Moreover, the Kelowna extractant demonstrated a 1:1 relationship and a high coefficient for $NO_3^-$ determination, and it correlated strongly with the Mehlich-3 extractant. However, it was 25 and 51% lower for P and K extractions, respectively. Therefore, it has been successfully applied to NPK ion-selective electrodes (ISE) for real-time soil nutrient analysis (Kim, 2006). In a recent study, the combination of Kelowna extraction with K-ISE showed a highly significant linear correlation compared

with the reference method, 1 M $NH_4OAc$ with AAS, for determining K content (Manatthammakul et al., 2023). Sarker et al. (2014) found that the Kelowna extractant exhibited weaker P extraction ability than Bray and Kurtz-1 and Mehlich-3 but was stronger than the Olsen method. Kelowna is an ideal extractant for potentiometric determination of soil P content using portable extractors (Sukaton et al., 2017).

### *Salt Extractant*

The 0.02 M strontium chloride–0.05 citric acid ($SrCl_2$–citrate) extractant, which is capable of extracting inorganic N, P, K, and other micronutrients, demonstrated significant efficacy in extracting P by showing strong correlations with the Olsen and Mehlich methods ($r > 0.97$) in acidic and alkaline soils (Simard et al., 1991). Subsequent studies revealed that $SrCl_2$–citrate exhibited slightly higher $NO_3^-$ and $NH_4^+$ extraction and strong correlations ($r > 0.70$) with the 2 M KCl extractant. It also showed a notably significant linear relationship with K extraction compared with 1 M $NH_4OAc$ and Mehlich-3 ($r > 0.90$) (Simard & Deschênes, 1992). Furthermore, $SrCl_2$–citrate extraction demonstrated higher K extraction than $SrCl_2$ and barium chloride ($BaCl_2$) extraction, demonstrating its ability to reliably predict the quantity of plant-available K in soil (Simard & Zizka, 1994). In another study, white precipitation was observed when the $SrCl_2$–citrate supernatant was combined with the Murphy and Riley method, a colorimetric P determination technique (Li et al., 2006).

In 2012, an evaluation of 0.01 M Ca chloride ($CaCl_2$), 0.02 M $SrCl_2$, and 0.01 M $BaCl_2$ as universal extractants concluded that 0.02 M $SrCl_2$ and 0.01 M $BaCl_2$ could serve as substitutes for conventional laboratory-based methods for determining available P across all soil types. For $NO_3^-$ analysis, 0.02 M $SrCl_2$ was suitable for basic soils, whereas 0.01 M $BaCl_2$ was optimal for acidic and neutral soils. However, compared with the 1 M $NH_4OAc$ method, these three universal extractants exhibited lower efficacy in extracting K (Bibiso et al., 2012). Moreover, among the evaluated universal extractants — 0.01 M $CaCl_2$, 0.01 M $BaCl_2$, 0.1 M $BaCl_2$, 0.02 M $SrCl_2$, Mehlich-3, and ammonium bicarbonate ($NH_4HCO_3$)–diethylenetriaminepentaacetic acid (DPTA)—0.02 M $SrCl_2$ exhibited the strongest and most significant correlation with standard laboratory-based methods for $NO_3^-$, P, K, Ca, and Mg across a soil pH range of 4.75–8.22 (Bibiso et al., 2015).

### *H3A Extractant*

Haney et al. (2006) introduced an H3A soil extractant comprising lithium citrate, citric acid, malic acid, oxalic acid, ethylenediaminetetraacetic acid (EDTA), and DTPA. Their study demonstrated that the H3A extractant exhibited a highly significant correlation with water and 1 M KCl ($r > 0.90$) for inorganic N extraction. Additionally, it showed significant correlations with water, Mechlich-3, and Olsen ($r > 0.61$) for extractable P across various soil conditions, including diverse clay contents, organic carbon levels, and

pH. The pH of the H3A extractant also significantly influences the extraction of P. Subsequently, H3A was modified to generate H3A-2 by eliminating EDTA and DTPA while adjusting the concentrations of lithium citrate, malic acid, oxalic acid, and citric acid at pH 4.4. This adaptation enhanced the estimation of inorganic N, P, K, Ca, and Zn, resulting in a clearer filtrate. It also significantly reduced the shaking time required for extraction from 30 min in the case of H3A-1 to a more efficient duration of just 5 min. H3A-2 demonstrated its ability to prevent overestimation or underestimation of soil P under various soil conditions. It also displayed significant correlations with water, Mechlich-3, Bray 1, and Olsen methods ($0.64 < r < 0.86$) for P, along with a strong correlation with 2 M KCl for inorganic N ($r = 0.97$) and $NH_4OC$ for K ($r = 0.95$) estimation (Haney et al., 2010). H3A-2 was further modified to produce H3A-3, composed of 0.006 M lithium citrate, 0.002 M citric acid, 0.004 M malic acid, and 0.003 M oxalic acid.

Evaluation using ferroaluminum oxide ($FeAlO_3$) strips, considered a "gold standard" for assessing plant-available P, revealed that H3A-3 yielded comparable results with <10% relative error and displayed a high correlation ($r = 0.98$) with $FeAlO_3$ strips. Consequently, H3A-3 has been proposed as a viable alternative method for determining plant-available P in soil, providing a cost-effective and time-saving solution compared with $FeAlO_3$ strip analysis (Haney et al., 2016). The following issues with lithium citrate cause turbidity in highly weathered soils with low pH and high Fe and Al concentrations, leading to complications in laboratory assays. Hence, researchers have attempted to eliminate lithium citrate from H3A-3. It resulted in the development of H3A-4, which consisted of 0.004 M citric acid, 0.008 M malic acid, and 0.005 M oxalic acid buffered at pH 3.75. The application involved using a soil-to-H3A-4 ratio of 1:10, followed by 10 min of shaking and 5 min of centrifugation to extract soil nutrients. H3A-4 notably produced a clear supernatant and maintained a strong linear relationship with H3A-3 for $NO_3^-$, $NH_4^+$, P, K, and Ca ($r > 0.80$) (Haney et al., 2017).

### *Universal Extractants for Alkaline- and Carbonate-rich Soils*

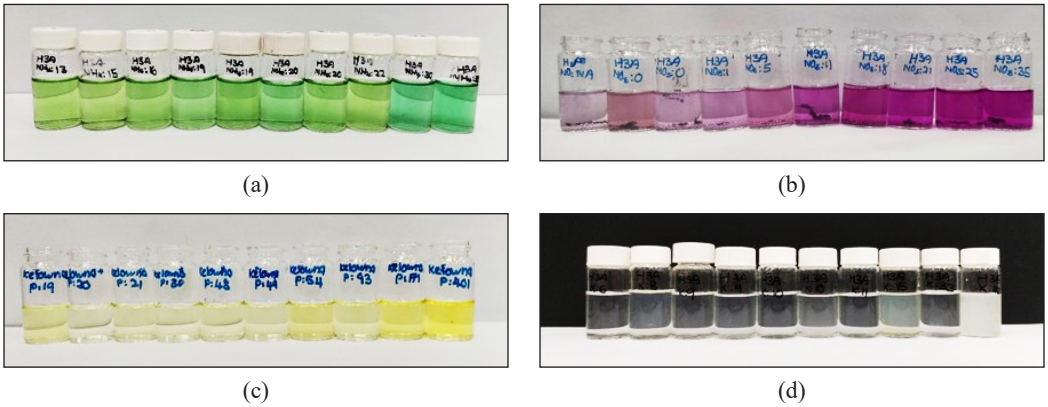
Previously, a 1 M $NH_4HCO_3$ and 0.005 M DTPA solution at pH 7.6 was used as a multiple-nutrient extractant for alkaline soils, which displayed high correlations exceeding 0.90 with phenoldisulfonic acid $NO_3^-$ test and Olsen P (Soltanpour & Schwab, 1977). More recently, a solution buffered at pH 8.5 consisting of 0.45 M sodium bicarbonate ($NaHCO_3$) and 0.374 M sodium sulfate ($Na_2SO_4$) was used to extract $NH_4^+$, P, and K from the calcareous soils of central China. This research demonstrated a strong linear relationship with standard laboratory methods using colorimetric reagents and a self-developed portable photoelectric system to determine $NH_4^+$, P, and K content ($r > 0.90$) (Ma et al., 2020).

## Colorimetric and Turbidimetric Systems

Pansu and Gautheyrou (2006) recommended various standard methods for determining soil nutrients, including steam distillation–titration and the indophenol blue reaction for $NH_4^+$ ion analysis; copper cadmium reduction with Griess reagent or $NO_3^-$ electrodes for $NO_3^-$ determination; molybdenum-based colorimetric methods, ICP, or AAS for P assessment; and flame photometry, AAS, or ICP spectrometry for K analysis. However, these methods require specialized machines and instruments, making them impractical for commercial use because of their initial cost, maintenance requirements, and technical complexity. Therefore, developing commercial NPK rapid test kits using colorimetric and turbidimetric methods is recommended. The description of color and turbidity is always subjective; hence, colorimetry and turbidimetry offer objective numerical systems to measure the color and cloudiness of a solution (Gilchrist & Nobbs, 1999). After nutrient extraction, these methods use specific reagents to visualize nutrient concentrations (Figure 1). It allowed for the estimation of nutrient content by visually comparing the color and turbidity of the solution to provide standard charts indicating low, moderate, and high concentrations (Table 1).

### *Ammonium*

Nessler's reagent was first described in 1856 (Neßler, 1856). In 1961, this method was introduced as a rapid testing method for soil and plant assays to measure $NH_4^+$ levels in small laboratories. However, certain interferences have raised turbidity issues, such as 5-ppm manganese, 500-ppm Mg, 5,000-ppm Ca, and 20-ppm Fe (Schuffelen et al., 1961). In the 2010s, a modified Nessler reagent was used in the wastewater industry, and it exhibited enhanced sensitivity and stability within the wavelength range of 400–425 nm (Jeong et al., 2013). This modified reagent has



(a) (b) (c) (d)

*Figure 1*. Variation in the color intensity of the H3A-4 soil extractant mixed with the simplifying Berthelot–salicylic method for $NH_4^+$ (a), the H3A-4 soil extractant mixed with the Zn–Griess reagent method for $NO_3^-$ (b), the Kelowna soil extractant mixed with the molybdenum yellow method for P (c), and the H3A-4 soil extractant mixed with the sodium tetraphenyl boron method for K (d), arranged from low to high concentrations

Table 1
*Guidelines for interpreting NPK levels in soil test results* (Horneck et al., n.d.)

| Nutrient concentration level | $NO_3^-$ (ppm) | P (Acidic soil) (ppm) | P (Alkaline soil) (ppm) | K (ppm) |
|---|---|---|---|---|
| Low | <10 | <20 | <10 | <150 |
| Medium | 10–20 | 20–40 | 10–20 | 150–250 |
| High | 20–30 | 40–100 | 20–40 | 250–800 |
| Excessive | >30 | >100 | >50 | >800 |

*Note*. $NH_4^+$ was excluded because it does not tend to accumulate in the soil due to its conversion to $NO_3^-$, usually within the 2–10 ppm range, and it can be higher under cold, wet conditions or in the presence of fertilizer residues

also been used in paper-based microfluidic devices to evaluate $NH_4^+$ levels in wastewater ranging from 0 to 5 ppm (Nxumalo et al., 2020). The nesslerization method is the standard method for $NH_4^+$ analysis of water and wastewater (American Public Health Association et al., n.d.).

Furthermore, the reaction of $NH_4^+$ with phenol and hypochlorite, which is catalyzed by Na nitroprusside to form blue indophenol, has been known as Berthelot's reaction or the phenol method since 1859 (Berthelot, 1859). $NH_4^+$ formed by the Kjeldahl digestion of soil and plant tissues has traditionally been determined using distillation–titration. The salicylic method is an alternative method that involves replacing phenol with Na salicylate. It is simpler and quicker, exhibiting over 99% recovery with precision and accuracy. This substitution was attributed to the carcinogenic nature of phenol (Baethgen & Alley, 1989). The salicylic method is recommended for total ammonia N (TAN) detection in aquaculture because it exhibits superior TAN concentrations, precision, and accuracy (Le & Boyd, 2012). Over time, a simplified version of the Berthelot – the salicylic method used a single reagent, achieving a 0.015-ppm $NH_4^+$ detection limit when combined with water samples and analyzed using an autonomous sensing platform at 660 nm (Cogan et al., 2014).

### *Nitrate*

Peter Griess, renowned for his pioneering work on diazo compounds, laid the foundation for significant advances in chemical analysis techniques and began his research in 1858 (Heines, 1958). Griess refined analytical methods over time, developing soil matrix spectrophotometric flow injection analysis (FIA) systems in 2007 and 2009. These systems precisely measure $NO_2^-$ and $NO_3^-$ levels by modifying the Griess–Ilosvay method. The process involves reducing $NO_3^-$ to $NO_2^-$ using Cd, followed by its reaction with Griess reagent to form a distinctive pink to reddish-purple azo dye complex, which is measured at 543 nm for accuracy (López Pasquali et al., 2007, 2010). Remarkably, the Cd reduction technique using the modified Griess–Ilosvay method remains the predominant method for determining $NO_3^-$ levels (Gelderman & Beegle, 2011).

However, because of the hazardous nature of Cd exposure, researchers have focused on finding alternatives. For example, vanadium chloride ($VCl_3$) has emerged as an alternative to Cd, generating a single $VCl_3$–Griess reagent that reduces toxic waste and simplifies its use. Nevertheless, this reagent shows a slow pink color development at room temperature (at least 4 hr) or when heated in a 60°C water bath for 2 hr (Doane & Horwáth, 2003). Additionally, the $VCl_3$–Griess reagent was successfully applied in automatic FIA to determine water samples, which showed no significant difference from the Cd column reduction method, and the recovery was above 89% (S.Wang et al., 2016).

Moreover, Zn reduction with Griess reagent has been proposed as a substitute for Cd reduction and high-performance liquid chromatography standard methods, showing no significant difference in $NO_2^-$ and $NO_3^-$ assays by running a paired t-test in food studies (Merino, 2009). Recently, a hydromonitrix $NO_3^-$ portable test kit using the Zn–Griess reagent was successfully developed to accurately detect 0.5–45 ppm $NO_3^-$ in freshwater within 10 min (Murray et al., 2017).

In summary, the colorimetric detection of $NO_2^-$ and $NO_3^-$ using the Griess reagent involves the reduction of $NO_3^-$ to $NO_2^-$ using either Cd, $VCl_3$, or Zn, followed by diazotization of $NO_2^-$ with sulfanilamide and its coupling with N-(1-naphthyl) ethylenediamine to form a pink–purple azo dye.

In parallel with these methods, the $NO_3^-$ salicylic acid method was introduced in 1975 for the rapid colorimetric determination of $NO_3^-$ in plant tissues. The sample was mixed with 5% salicylic acid in concentrated sulfuric acid and 2 N sodium hydroxide (NaOH), resulting in a yellow solution detected at 410 nm (Cataldo et al., 1975). In 1990, researchers demonstrated that $NO_3^-$ salicylic acid and Cd reduction methods obtained similar results for soil $NO_3^-$ ($r$ = 0.99) (Vendrell & Zupancic, 1990). The International Organization for Standardization (2020) has standardized salicylic acid as a colorimetric reagent for water-soluble $NO_3^-$ determination.

### *Phosphorus*

Murphy and Riley's technique, also known as the molybdenum blue (MB) method, was originally designed to measure soluble phosphate levels in seawater. It is a single-color reagent composed of ammonium molybdate, K antimony tartrate, ascorbic acid, and sulfuric acid, which quickly develops a bluish-purple color in the presence of phosphate and is detectable at 882 nm (Murphy & Riley, 1962). MB reactions involve a sequence of complex chemical processes that convert orthophosphate ($PO_4^-$) into phosphomolybdenum blue species, which require a strong acid molybdenum source and reducing agent. The MB method is widely used in analytical chemistry (Nagul et al., 2015).

The vanadomolybdophosphoric acid or molybdenum yellow (MY) method was

introduced in 1971 (Kaylor, 1971). When implementing P monitoring via microsystem technology (MST), the MB method was disregarded because of the reagent's short shelf life and tendency to form small precipitates, leading to channel blockages in microfluidic systems and cuvette wall coatings. Instead, the researchers found that the MY method was compatible with MST. It offered the following advantages: it did not precipitate, maintained its performance after a year of storage, and operated effectively under a spectrum of 380 nm absorbance. This method successfully determined P within the 0–50 ppm range, with a remarkable detection limit of 0.2 ppm (Bowden et al., 2002). Furthermore, the MY method was successfully implemented in a microfluidic paper-based analytical device to evaluate P in water (Waghwani et al., 2019).

***Potassium***

Sodium cobaltinitrite ($CoN_6Na_3O_{12}$) reacts with K to form potassium sodium cobaltinitrite ($K_2NaCo(NO_2)_6 \cdot H_2O$) precipitate, which was used in 1933 to rapidly determine K levels in plant tissues (Morris & Gerdel, 1933). In 1950, the same $CoN_6Na_3O_{12}$ reagent was used for K determination in soil extracts, forming a methyl orange cobaltinitrite precipitate (Whittles & Little, 1950).

The discovery of Na tetraphenyl boron ($Na(C_6H_5)_4B$) in 1949, which forms a white insoluble compound with K ions in NaOH solution, presented a more effective method for determining the K value of Morgan-extracted soil than the flame test, especially in samples with elevated Na levels and minimal interference from $NH_4^+$ ions. The turbidimetric reagents were composed of 1.2% $Na(C_6H_5)_4B$ and mixed with aluminium hydroxide powder, 40% NaOH, and formaldehyde solutions to avoid interference from impurities such as $NH_4^+$ (Paul & Gibson Jr., 1959; Ståhlberg, 1979). An integrated approach combining colorimetric NPK determination with irrigation decision-making technology has recently emerged. This system uses a $Na(C_6H_5)_4B$ turbidimetric reagent incorporating EDTA disodium salt, sodium tetraborate solution, and formaldehyde as masking agents. This method accurately determined K in compound NPK fertilizers with an error margin of <5% at 430 nm (Liu et al., 2021).

Similarly, in medical research, the detection of K in urine samples was performed using crown ether-modified gold (Au) nanoparticles (Au NPs) using either a colorimetric sensor with an absorption ratio of A620 nm/A520 nm or paper-based colorimetric array test strips. Rapid color formation involves the reaction of the crown ester (4-aminobenzo-18-crown-6, ABC) with $K^+$ ions to form a 2:1 stable sandwich complex — the amino groups of the ABC–$K^+$ complex and Au NPs form Au–N bonds. The color of the wine-red Au NP solution changed to violet or gray when $K^+$ was present (Chitbankluai et al., 2021; Qiu et al., 2019).

## DISCUSSION

The swift development of soil test kits has commercialized the rapid and precise determination of soil nutrient concentrations. Extensive research has been conducted on universal extractants and colorimetric and turbidimetric systems for determining nutrient content. However, published protocols are based on laboratory routines and often lack direct applicability to rapid commercial test kits. Nonetheless, these studies serve as valuable guidelines for adaptation and highlight crucial criteria for further research into rapid test kit development. Some pivotal criteria include ensuring the suitability of the universal extractant for various local soil conditions, ease of convenient use, avoidance of hazardous chemicals, compatibility of the extractant with colorimetric and turbidimetric systems, and long shelf life. These considerations form the cornerstone for successfully developing rapid soil test kits in the commercial sphere.

### Criterion 1: Suitability of Universal Extractant under Various Local Soil Conditions

Soil can be categorized as acidic, alkaline, calcareous, or noncalcareous. Researchers commonly used 2 M KCl for $NH_4^+$ and $NO_3^-$, along with $NH_4OAc$ for $K^+$, to validate the performance of the universal extractant. However, variations in the P extraction methods, such as Olsen and Bray, were adopted because of the soil pH and calcareous characteristics. The Food and Agriculture Organization of the United Nations (FAO) (2021a, 2021b) delineated the suitability of Bray for soil pH below 6.8 and Olsen for calcareous and alkaline soils. Notably, Bray II extraction in alkaline soil tends to overestimate P because of its acidity, potentially neutralizing or excessively extracting P from calcium phosphate. The optimal P extraction principles were mirrored in the Morgan extractant buffered at 4.8 for acidic soil and 0.45 M $NaHCO_3$ + 0.374 M $Na_2SO_4$ buffered at 8.5 solution for alkaline and calcareous soils.

In contrast, H3A-4 and Kelowna can be used in all types of soils. H3A, which contains artificial organic root-exclusion compounds, fosters an actively rooted rhizosphere by simulating organic acid root exudates to alleviate plant nutrient deficiencies (Haney et al., 2006). Although Morgan and Kelowna contained HOAc, Kelowna's lower HOAc normality and shorter shaking time helped prevent excessive P extraction under calcareous soil conditions (Table 2).

### Criterion 2: Ease of Use

The universal extractant, colorimetric and turbidimetric methods typically require a mechanical shaker and a spectrometer, which poses challenges in field handling. Many studies using universal extractants used shaking for at least 5 min to ensure homogeneous extraction (Table 2). Manual vigorous shaking for 1 min is recommended to adapt to field conditions. However, shorter time and nonuniform shaking

speed may affect nutrient extraction efficiency. Colorimetric and turbidimetric methods have been developed using portable spectrometers, FIA, paper-based microfluidic devices, and MST (Bowden et al., 2002; Liu et al., 2021; Ma et al., 2020; S. Wang et al., 2016; Waghwani et al., 2019). However, these innovations are geared toward rapid professional testing rather than commercial testing because of high investment costs, the need for skilled technicians, and high maintenance costs.

A standardized color chart for identifying soil nutrient deficiency, sufficiency, and excess is sufficient for commercial use. Additionally, quicker color changes and turbid forms are preferable. For instance, the Zn–Griess reagent is superior to the $VCl_3$–Griess reagent because its color is formed within 10 min. Furthermore, rapid soil test kits must strongly correlate with standard laboratory-based methods to ensure accuracy. By referring to approximate or categorical nutrient concentration standards, these kits can offer fertilization recommendations tailored to local commercial crops and promote sustainable farming practices.

**Criterion 3: Avoid Hazardous Chemicals**

The rapid test kit is intended for commercial use. Users may not be professionals when handling chemicals; hence, dangerous components must be avoided. As shown in Table 3, formulations containing strongly corrosive, hazardous, or carcinogenic compounds, such as $NO_3^-$ salicylic acid, $NH_4^+$ Nessler, and phenol methods, are not recommended because of the potential dangers to users and the risk of environmental pollution.

**Criterion 4: Compatibility of Extractants with Colorimetric and Turbidimetric Systems**

Most studies on universal extractants and nutrient determination using colorimetry and turbidimetry have been conducted independently. Therefore, the compatibility of the universal extractant (Table 2) with the colorimetric and turbidimetric reagents (Table 3) is unclear. For example, when mixed using the Murphy and Riley method, a 0.02 M $SrCl_2$–0.05 citric acid extractant obstructs color formation (Li et al., 2006). Therefore, it is essential to evaluate compatibility to facilitate the development of rapid test kits.

**Criterion 5: Long Shelf Life**

Commercialized rapid soil test kits must account for their shelf life. Unlike in laboratory settings, where extractants and reagents can be freshly prepared as needed, the extended longevity of these reagents requires further investigation. Prolonged storage may alter extraction efficiency and sensitivity to color development. For example, ascorbic acid in MB has limited stability and requires daily preparation (Bowden et al., 2002; FAO, 2021b). The ideal shelf life of rapid test kits is at least one year. Extensive research on the stability and preservation of these components is crucial for ensuring their effectiveness and accuracy over extended periods.

Table 2
*Universal extractants' composition, pH buffer, nutrient extraction, suitable soil, shaking time, and soil extraction ratio*

| Universal extractant | Composition | pH buffer | Extracted nutrient (NPK) | Suitable soil type | Shaking time | Soil: extraction ratio | Reference |
|---|---|---|---|---|---|---|---|
| Morgan | 0.73 M $C_2H_3NaO_2$ and 0.52 N HOAc | 4.8 | $NH_4^+$, $NO_3^-$, P, and K | Acidic soil | 15 min | 1:4 | Morgan (1941) |
| 0.02 M $SrCl_2$ | 0.02 M $SrCl_2$ | | $NO_3^-$, P, and K | Basic and calcareous soils | 30 min | 1:10 | Bibiso et al. (2012) |
| 0.01 M $BaCl_2$ | 0.01 M $BaCl_2$ | | $NO_3^-$, P, and K | Acidic and neutral soils | 30 min | 1:10 | Bibiso et al. (2015) |
| H3A-4 | 0.004 M citric acid + 0.008 M malic acid + 0.005 M oxalic acid | 3.75 | $NH_4^+$, $NO_3^-$, P, and K | All types of soil | 10 min shaking + 5 min centrifuge | 1:10 | Haney et al. (2017) |
| Kelowna | 0.25 N HOAc + 0.015 N $NH_4F$ | | $NH_4^+$, $NO_3^-$, P, and K | All types of soil | 5 min | 1:10 | van Lierop (1988) |
| Ma et al. universal extractant | 0.45 M $NaHCO_3$ + 0.374 M $Na_2SO_4$ | 8.5 | $NH_4^+$, $NO_3^-$, P, and K | Neutral and calcareous soil | 10 min | 1:20 | Ma et al. (2020) |

*Note.* $C_2H_3NaO_2$ = Sodium acetate; HOAc = Acetic acid; $SrCl_2$ = Strontium chloride; $BaCl_2$ = Barium chloride; $NH_4F$ = Ammonium fluoride; $NaHCO_3$ = Sodium bicarbonate; $Na_2SO_4$ = Sodium sulfate

Table 3
*The color form, absorbance wavelength, and limitation of colorimetric and turbidimetric reagents*

| Nutrient | Method | Color form | Absorbance wavelength (nm) | Limitation | Reference |
|---|---|---|---|---|---|
| $NH_4^+$ | Modified Nessler reagent | Yellow-brown | 400–425 | Contains mercury (II) iodide as a hazardous element | Jeong et al. (2013) |
| | Phenol method | Indophenol blue | | Contains phenol as a carcinogenic compound | Berthelot (1859) |
| | Simplifying Berthelot–salicylic method | Green | 660 | Not yet tested with other universal extractants | Cogan et al. (2014) |
| $NO_3^-$ | Salicylic acid method | Yellow | 410 | Contains concentrated sulfuric acid as a corrosive chemical | Cataldo et al. (1975) Vendrell and Zupancic (1990) |
| | Modified Griess–Ilosvay method | Pink | 543 | Contains cadmium as a carcinogenic chemical | López Pasquali et al. (2007, 2010) |
| | $VCl_3$–Griess reagent | Pink | 543 | Requires at least 4 hr of color formation at room temperature | Doane and Horwáth (2003) |
| | Zn–Griess reagent | Pink | 543 | Not yet tested with other universal extractants | Murray et al. (2017) |
| P | Molybdenum blue | Blue | 883 | Short shelf life | Murphy and Riley (1962) |
| | Molybdenum yellow | Yellow | 400 | Not yet tested with other universal extractants | Kaylor (1971) |
| K | Sodium cobaltinitrite | Yellow precipitate | | Relatively not precise, sensitive, and user-friendly | Whittles and Little (1950) |
| | Sodium tetraphenyl boron method | White precipitate | 430 | Not yet tested with other universal extractants | Liu et al. (2021) |
| | Crown ether-modified gold particles | Violet or gray | 620 | Expensive | Qiu et al. (2019) |

As rapid soil test kits have advanced to meet the demands of modern agriculture, they offer significant advantages in terms of affordability, accessibility, and quick results. However, in addition to these benefits, inherent limitations require careful consideration.

## Advantages of Rapid Soil Test Kits for Agricultural Practices

### *Advantage 1: Lower Price*

Farmers in tropical countries, such as Nigeria, the Democratic Republic of Congo, Ethiopia, India, and Bangladesh, face significant challenges that hinder their productivity. These constraints make it difficult for these people to fully comprehend soil profiles, which are essential for successfully growing crops and improving livelihoods. The State of the Tropics 2020 Report highlights the extreme poverty prevalent in these regions and the often-limited infrastructure (James Cook University [JCU], 2020). It makes wet chemistry analysis for soil testing unaffordable for impoverished farmers. Consequently, alternative soil testing methods, such as rapid nutrient testing, are being deployed in Africa, offering more affordable rates (Dimkpa et al., 2017).

### *Advantage 2: In situ Soil Testing/Higher Accessibility*

The traditional approach encompasses a series of detailed steps for farms aiming to perform soil nutrient analysis. Initially, soil samples are collected and dispatched to a laboratory, where they are dried, sieved to a standard size of 2 mm or smaller, and then subjected to nutrient extraction using specific chemicals. Specialized equipment, such as ICP and AAS, is used to quantify the extracted nutrients. This procedure, culminating in calculating soil nutrient levels, is intricate and time-intensive, presenting challenges for those not specialized in the field.

In contrast, rapid soil test kits offer a more streamlined and user-friendly alternative. These kits bypass the need to send samples to a laboratory, allowing on-site nutrient analysis. Users can easily determine the soil nutrient content by comparing the color saturation of the test with that of the provided color chart, which yields immediate results. This efficiency enables farmers to rapidly evaluate their soil nutrient status and develop appropriate nutrient management strategies to enhance agricultural productivity and sustainability.

### *Advantage 3: Instantaneous or Faster Results*

Traditional vegetable farmers, particularly those dependent on their land for income, frequently bypass wet chemistry analysis to determine soil nutrients because of the lengthy duration. This reluctance is especially pronounced in areas with very short crop cycles, sometimes as short as 25 days. Such rapid turnaround times require swift decision-making regarding fertilization and overall crop management to maximize yield and profitability. The standard wet nutrient chemistry analysis process, which can span several days to

months, is impractical for these farmers. Immediate information is required to prepare fertilization strategies for imminent planting cycles. Therefore, instant soil nutrient testing methods are indispensable for these farmers, offering prompt and practical insights to facilitate effective crop management under the constraints of short crop cycles.

## Limitations of Rapid Soil Test Kits

Although rapid soil test kits offer an accessible solution for soil nutrient analysis in resource-limited regions at a lower cost and provide instant *in situ* results, they cannot entirely replace standard wet chemistry analysis for several reasons.

### *Limitation 1: User Dependency and Error*

The accuracy and reliability of the results obtained from rapid soil test kits depend heavily on user proficiency. Improper sampling techniques, inadequate mixing of soil and reagents, and misinterpreting colorimetric readings can lead to erroneous results. Without adherence to standardized protocols, users may obtain inconsistent or unreliable results, emphasizing the importance of user proficiency and protocol adherence to ensure the accuracy of rapid soil test kit results.

### *Limitation 2: Interference with Soil Properties*

Soil properties, including texture, organic matter content, pH, and mineral composition, are dynamic factors that can significantly affect the performance of rapid soil test kits. These variations can introduce interference during testing and lead to inaccurate results. For instance, H3A-3 has exhibited turbidity issues in soils with low pH and high Fe and Al concentrations (Haney et al., 2017). Additionally, the suitability of universal extractants for various local soil conditions must be considered, as shown in Table 1. Different soil types require different extraction methods and reagents to ensure accurate nutrient analysis.

### *Limitation 3: Result Interpretation*

Rapid soil test kits categorize soil NPK levels to determine appropriate fertilization strategies. These strategies vary with crop type, soil texture, and climate. However, some farmers may struggle to interpret the results effectively to enhance soil fertility. Therefore, soil test kits should include fertilization recommendations tailored to different soil conditions and crops to aid farmers in making informed decisions regarding optimal soil management and crop productivity.

### *Limitation 4: Validation Challenges*

The reliability of commercially available rapid soil test kits is often under scrutiny because of a significant lack of validation, which is a concern underscored by Dimkpa et al. (2017). Furthermore, the validation of several commercial test kits has been documented in only one study based on outdated information from Faber et al. (2007). Our literature review revealed a scarcity of comprehensive

discussions on the complete NPK rapid test kit methodology, with most research focusing on individual aspects, such as universal extractants and colorimetric and turbidimetric reagents. This fragmentation hinders the synthesis of the research findings. Therefore, it is essential to conduct further validation studies of the latest developments in rapid soil test kits to confirm their reliability and accuracy for effective use.

### *Limitation 5: Accuracy and Precision*

The effectiveness of rapid soil test kits in delivering accurate and precise results is hindered by several factors, such as dependence on the user's skill, interference from unique soil properties, challenges in interpreting results, and validation constraints. Despite technological progress, these kits may not always provide reliable results because of soil analysis's complex nature and inherent limitations. Moreover, despite being commercially available, these kits have not yet reached maturity in terms of development and validation. Overcoming these obstacles is crucial for improving the dependability and utility of rapid soil test kits for accurate nutrient evaluations, thereby supporting optimal agricultural practices.

Given these considerations, rapid test kits and standard wet chemistry analyses should ideally be used as supplementary instruments. The latter continues to be essential for thoroughly assessing and managing soil fertility, underscoring the importance of integrating both methods to achieve a comprehensive approach to soil health and crop management.

## CONCLUSION

The development of rapid *in situ* soil NPK test kits revolutionizes conventional, expensive, and time-consuming laboratory methods. These kits rely on universal extractants for multiple soil nutrient extractions and use colorimetric and turbidimetric reagents for nutrient determination. For commercial viability, these kits, along with standardized categorical value color charts, must embody the traits of speed, accuracy, durability, simplicity, affordability, and safety.

Among the options, the inclusion of H3A-4 or Kelowna coupled with Zn using Griess reagent for $NO_2^-$ and $NO_3^-$, salicylic method for $NH_4^+$, molybdenum yellow method for P, and $Na(C_6H_5)_4B$ for K emerged as the most optimal development choices. H3A-4 and Kelowna demonstrated versatility across soil types, whereas the other reagents offered longer shelf life, safety, quicker color formation, and fewer reported compatibility issues. Refining these rapid test kits requires meticulous testing and validation against standard laboratory methods to determine their accuracy before widespread application. Patience and thorough validation are critical steps in model development.

## ACKNOWLEDGEMENTS

This study was funded by Universiti Putra Malaysia under Geran Putra Inisiatif Siswazah (GP-IPS), with the project code GP-IPS/2023/9769660.

## REFERENCES


American Public Health Association, American Water Works Association, & Water Environment Federation. (n.d.). *Standard methods for the examination of water and wastewater: 4500-$NH_3$ (ammonia)*. APHA Press.

Baethgen, W. E., & Alley, M. M. (1989). A manual colorimetric procedure for measuring ammonium nitrogen in soil and plant Kjeldahl digests. *Communications in Soil Science and Plant Analysis*, *20*(9–10), 961–969. https://doi.org/10.1080/00103628909368129

Berthelot, M. P. E. (1859). *Berthelot's reaction mechanism*. Report de Chimie Applique.

Bibiso, M., Taddesse, A. M., & Assefa, M. (2012). Evaluation of three universal extractants for the determination of P, $NO_3^-$ and K in some soils of Ethiopia. *Advances in Life Science and Technology*, *6*, 16–24.

Bibiso, M., Taddesse, A. M., Gebrekidan, H., & Melese, A. (2015). Evaluation of universal extractants for determination of some macronutrients from soil. *Communications in Soil Science and Plant Analysis*, *46*(19), 2425–2448. https://doi.org/10.1080/00103624.2015.1081925

Bowden, M., Sequiera, M., Krog, J. P., Gravesen, P., & Diamond, D. (2002). Analysis of river water samples utilising a prototype industrial sensing system for phosphorus based on micro-system technology. *Journal of Environmental Monitoring*, *4*(5), 767–771. https://doi.org/10.1039/b200330a

Bünemann, E. K., Bongiorno, G., Bai, Z., Creamer, R. E., Deyn, G. D., de Goede, R., Fleskens, L., Geissen, V., Kuyper, T. W., Mäder, P., Pulleman, M., Sukkel, W., van Groenigen, J. W., & Brussaard, L. (2018). Soil quality — A critical review. *Soil Biology and Biochemistry*, *120*, 105–125. https://doi.org/10.1016/j.soilbio.2018.01.030

Cataldo, D. A., Maroon, M., Schrader, L. E., & Youngs, V. L. (1975). Rapid colorimetric determination of nitrate in plant-tissue by nitration of salicylic-acid. *Communications in Soil Science and Plant Analysis*, *6*(1), 71–80. https://doi.org/10.1080/00103627509366547

Chitbankluai, K., Buranachai, C., Limbut, W., & Thavarungkul, P. (2021). Paper-based colorimetric sensor for potassium ion detection in urine by crown ether modified gold nanoparticles. *Journal of Physics: Conference Series* (Vol. 1719, No. 1, p. 012026). IOP Publishing. https://doi.org/10.1088/1742-6596/1719/1/012026

Cogan, D., Cleary, J., Fay, C., Rickard, A., Jankowski, K., Phelan, T., Bowkett, M., & Diamond, D. (2014). The development of an autonomous sensing platform for the monitoring of ammonia in water using a simplified Berthelot method. *Analytical Methods*, *6*(19), 7606–7614. https://doi.org/10.1039/C4AY01359J

De Silva, C. S., Koralage, I. S. A., Weerasinghe, P., & Silva, N. R. N. (2015). The determination of available phosphorus in soil: A quick and simple method. *OUSL Journal*, *8*, 1–7. https://doi.org/10.4038/ouslj.v8i0.7315

Dimkpa, C., Bindraban, P., McLean, J. E., Gatere, L., Singh, U., & Hellums, D. (2017). Methods for rapid testing of plant and soil nutrients. In E. Lichtfouse (Ed.), *Sustainable agriculture reviews* (Vol. 25, pp. 1–43). Springer. https://doi.org/10.1007/978-3-319-58679-3_1

Doane, T. A., & Horwáth, W. R. (2003). Spectrophotometric determination of nitrate with a single reagent. *Analytical Letters*, *36*(12), 2713–2722. https://doi.org/10.1081/AL-120024647

Faber, B. A., Downer, A. J., Holstege, D., & Mochizuki, M. J. (2007). Accuracy varies for commercially available soil test kits analyzing nitrate–nitrogen, phosphorus, potassium, and

pH. *HortTechnology*, *17*(3), 358-362. https://doi.org/10.21273/HORTTECH.17.3.358

Food and Agriculture Organization of the United Nations. (2021a). *Standard operating procedure for soil available phosphorus: Bray I and Bray II method*. FAO. https://www.fao.org/3/cb3460en/cb3460en.pdf

Food and Agriculture Organization of the United Nations. (2021b). *Standard operating procedure for soil available phosphorus: Olsen method*. FAO. https://www.fao.org/3/cb3644en/cb3644en.pdf

Gelderman, R. H., & Beegle, D. (2011). Nitrate-nitrogen. In J. R. Brown (Ed.), *Recommended chemical soil test procedures: For the north central region* (pp. 17–20). Missouri Agricultural Experiment Station.

Gilchrist, A., & Nobbs, J. (1999). Colorimetry, theory. In J. Lindon, J. Holmes, & G. Tranter (Eds.), *Encyclopedia of spectroscopy and spectrometry* (pp. 337–343). Academic Press. https://doi.org/10.1006/rwsp.2000.0044

Gough, N. (1996). *Soil and plant tissue testing method and interpretations of their results for Bristish Columbia agricultural soils*. https://www2.gov.bc.ca/assets/gov/farming-natural-resources-and-industry/agriculture-and-seafood/agricultural-land-and-environment/soil-nutrients/600-series/634200-3_soil_and_plant_tissue_testing_methods.pdf

Hachiya, T., & Sakakibara, H. (2016). Interactions between nitrate and ammonium in their uptake, allocation, assimilation, and signaling in plants. *Journal of Experimental Botany*, *68*(10), 2501–2512. https://doi.org/10.1093/jxb/erw449

Haney, R. L., Haney, E. B., Harmel, R. D., Smith, D. R., & White, M. J. (2016). Evaluation of H3A for determination of plant available P vs. FeAlO strips. *Open Journal of Soil Science*, *6*(11), 175–187. https://doi.org/10.4236/ojss.2016.611017

Haney, R. L., Haney, E. B., Hossner, L. R., & Arnold, J. G. (2006). Development of a new soil extractant for simultaneous phosphorus, ammonium, and nitrate analysis. *Communications in Soil Science and Plant Analysis*, *37*(11–12), 1511–1523. https://doi.org/10.1080/00103620600709977

Haney, R. L., Haney, E. B., Hossner, L. R., & Arnold, J. G. (2010). Modifications to the new soil extractant H3A-1: A multinutrient extractant. *Communications in Soil Science and Plant Analysis*, *41*(12), 1513–1523. https://doi.org/10.1080/00103624.2010.482173

Haney, R. L., Haney, E. B., Smith, D. R., & White, M. J. (2017). Removal of lithium citrate from H3A for determination of plant available P. *Open Journal of Soil Science*, *7*(11), 301–314. https://doi.org/10.4236/ojss.2017.711022

Heines, S. V. (1958). Peter Griess — Discoverer of diazo compounds. *Journal of Chemical Education*, *35*(4), 187. https://doi.org/10.1021/ed035p187

Horneck, D. A., Sullivan, D. M., Owen, J., & Hart, J. M. (n.d.). *Soil test interpretation guide*. Oregon State University Extension Service. https://extension.oregonstate.edu/sites/default/files/catalog/auto/EC1478.pdf

International Organization for Standardization. (2020). *General methods of test for pigments — Part 19: Determination of water-soluble nitrates (salicylic acid method)*. ISO. https://www.iso.org/obp/ui/en/#iso:std:iso:787:-19:ed-2:v1:en

James Cook University. (2020). *State of the tropics 2020 report*. JCU. https://www.jcu.edu.au/__data/assets/pdf_file/0004/1271956/State-of-the-Tropics-2020-Summary.pdf

Jeong, H., Park, J., & Kim, H. (2013). Determination of $NH_4^+$ in environmental water with interfering substances using the modified Nessler method. *Journal of Chemistry*, *2013*, 359217. https://doi.org/10.1155/2013/359217

Jones Jr., J. B. (1990). Universal soil extractants: Their composition and use. *Communications in Soil Science and Plant Analysis*, *21*(13–16), 1091–1101. https://doi.org/10.1080/00103629009368292

Kaylor, W. H. (1971). *Determination of the phosphate in solid waste using the vanadomolybdophosphoric acid method*. U.S. Environmental Protection Agency.

Kim, H.-J. (2006). *Ion-selective electrodes for simultaneous real-time analysis for soil macronutrients* [Doctoral dissertation, University of Missouri-Columbia]. MOspace. https://mospace.umsystem.edu/xmlui/bitstream/handle/10355/4471/research.pdf?sequence=3

Kumawat, C., Yadav, B., Verma, A. K., Meena, R. K., Pawar, R., Kharia, S. K., Yadav, R. K., Bajiya, R., Pawar, A., Sunil, B. H., & Trivedi, V. (2017). Recent Developments in multi-nutrient extractants used in soil analysis. *International Journal of Current Microbiology and Applied Sciences*, *6*(5), 2578–2584. https://doi.org/10.20546/ijcmas.2017.605.290

Le, P. T. T., & Boyd, C. E. (2012). Comparison of phenate and salicylate methods for determination of total ammonia nitrogen in freshwater and saline water. *Journal of the World Aquaculture Society*, *43*(6), 885–889. https://doi.org/10.1111/j.1749-7345.2012.00616.x

Li, L., Zhang, J., Xing, W., Chen, W., Wu, X., & Zhu, K. (2006). Development and validation of a new soil universal extractant: 0.02 molar strontium chloride. *Communications in Soil Science and Plant Analysis*, *37*(11–12), 1627–1638. https://doi.org/10.1080/00103620600710249

Liu, N., Wei, Z., & Wei, H. (2021). Colorimetric detection of nitrogen, phosphorus, and potassium contents and integration into field irrigation decision technology. *IOP Conference Series: Earth and Environmental Science* (Vol. 651, No. 4, p. 042044). IOP Publishing. https://doi.org/10.1088/1755-1315/651/4/042044

López Pasquali, C. E., Fernández Hernando, P., & Durand Alegría, J. S. (2007). Spectrophotometric simultaneous determination of nitrite, nitrate and ammonium in soils by flow injection analysis. *Analytica Chimica Acta*, *600*(1–2), 177–182. https://doi.org/10.1016/j.aca.2007.03.015

López Pasquali, C. E., Gallego-Picó, A., Fernández Hernando, P., Velasco, M., & Durand Alegría, J. S. (2010). Two rapid and sensitive automated methods for the determination of nitrite and nitrate in soil samples. *Microchemical Journal*, *94*(1), 79–82. https://doi.org/10.1016/j.microc.2009.09.005

Ma, L., Duan, T., & Hu, J. (2020). Application of a universal soil extractant for determining the available NPK: A case study of crop planting zones in central China. *Science of The Total Environment*, *704*, 135253. https://doi.org/10.1016/j.scitotenv.2019.135253

Manatthammakul, W., Chittamart, N., Tawornpruek, S., & Aramrak, S. (2023). Correlation of soil available potassium rapidly analyzed by ion selective electrode and extracted by soil potassium extractants. *Khon Kaen Agriculture Journal*, *51*(4), 634–647. https://doi.org/10.14456/kaj.2023.48

Masjkur, M. (2009). Correlation between soil test phosphorus of kaolinitic and smectitic soils with phosphorus uptake of lowland rice. *Journal of Tropical Soils*, *14*(3), 205–209. https://doi.org/10.5400/jts.2009.v14i3.205-209

McIntosh, J. L. (1969). Bray and Morgan soil extractants modified for testing acid soils from different parent materials. *Agronomy Journal*, *61*(2), 259–265. https://doi.org/10.2134/agronj1969.00021962006100020025x

Merino, L. (2009). Development and validation of a method for determination of residual nitrite/nitrate in foodstuffs and water after zinc reduction. *Food Analytical Methods*, *2*, 212–220. https://doi.org/10.1007/s12161-008-9052-1

Morgan, M. P. (1941). *Chemical soil diagnosis by the universal soil testing system (a revision of Bulletin 392)*. Connecticut Experiment Station. https://portal.ct.gov/-/media/caes/documents/publications/bulletins/b450pdf.pdf?la=en

Morris, V. H., & Gerdel, R. W. (1933). Rapid colorimetric determination of potassium in plant tissues. *Plant Physiology*, *8*(2), 315–319. https://doi.org/10.1104/pp.8.2.315

Murphy, J., & Riley, J. P. (1962). A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*, *27*, 31–36. https://doi.org/10.1016/S0003-2670(00)88444-5

Murray, E., Nesterenko, E. P., McCaul, M., Morrin, A., Diamond, D., & Moore, B. (2017). A colorimetric method for use within portable test kits for nitrate determination in various water matrices. *Analytical Methods*, *9*(4), 680–687. https://doi.org/10.1039/C6AY03190K

Nagul, E. A., McKelvie, I. D., Worsfold, P., & Kolev, S. D. (2015). The molybdenum blue reaction for the determination of orthophosphate revisited: Opening the black box. *Analytica Chimica Acta*, *890*, 60–82. https://doi.org/10.1016/j.aca.2015.07.030

Neßler, J. (1856). *Über das verhalten des jodquecksilbers und der quecksilberverbindungen überhaupt zu ammoniak und über eine neue reaction auf ammoniak* [On the behaviour of iodine mercury and mercury compounds in general to ammonia and on a new reaction to ammonia]. Poppen.

Nxumalo, N. L., Madikizela, L. M., Kruger, H. G., Onwubu, S. C., & Mdluli, P. S. (2020). Development of a paper-based microfluidic device for the quantification of ammonia in industrial wastewater. *Water SA*, *46*(3), 506–513. https://doi.org/10.17159/wsa/2020.v46.i3.8661

Pansu, M., & Gautheyrou, J. (2006). *Handbook of soil analysis: Mineralogical, organic and inorganic methods*. Springer. https://doi.org/10.1007/978-3-540-31211-6

Paul, A. D., & Gibson Jr., J. A. (1959). Qualitative test for potassium using sodium tetraphenylboron. *Journal of Chemical Education*, *36*(8), 380. https://doi.org/10.1021/ed036p380

Qiu, J., Zhang, Y., Dong, C., Huang, Y., Sun, L., Ruan, H., Wang, H., Li, X., & Wu, A. (2019). Rapid colorimetric detection of potassium ions based on crown ether modified Au NPs sensor. *Sensors and Actuators B: Chemical*, *281*, 783–788. https://doi.org/10.1016/j.snb.2018.10.139

Ritchie, H. (2021). *Excess fertilizer use: Which countries cause environmental damage by overapplying fertilizers?* Our World in Data. https://ourworldindata.org/excess-fertilizer

Roobroeck, D., Van Asten, P. J. A., Jama, B., Harawa, R., & Vanlauwe, B. (2016). *Integrated soil fertility management: Contributions of framework and practices to climate-smart agriculture*. https://www.researchgate.net/publication/289962704_Integrated_Soil_Fertility_Management_Contributions_of_framework_and_practices_to_Climate-Smart_Agriculture?channel=doi&linkId=5693ad8808aeab58a9a29f3d&showFulltext=true

Sahrawat, K. L. (1979). Evaluation of some chemical extractants for determination of exchangeable ammonium in tropical rice soils. *Communications in Soil Science and Plant Analysis*, *10*(7), 1005–1013. https://doi.org/10.1080/00103627909366957

Sahrawat, K. L., & Prasad, R. (1975). A rapid method for determination of nitrate, nitrite, and ammoniacal nitrogen in soils. *Plant and*

*Soil*, *42*, 305–308. https://doi.org/10.1007/BF02186992

Sarker, A., Kashem, M. A., Osman, K. T., Hossain, I., & Ahmed, F. (2014). Evaluation of available phosphorus by soil test methods in an acidic soil incubated with different levels of lime and phosphorus. *Open Journal of Soil Science*, *4*(3), 103–108. https://doi.org/10.4236/ojss.2014.43014

Schuffelen, A. C., Muller, A., & Van Schouwenburg, J. C. (1961). Quick-tests for soil and plant analysis used by small laboratories. *Netherlands Journal of Agricultural Science*, *9*(1), 2–16. https://doi.org/10.18174/njas.v9i1.17630

Simard, R. R., & Deschênes, M. (1992). Strontium chloride-citric acid extraction procedure for agricultural and environmental purposes. *Communications in Soil Science and Plant Analysis*, *23*(17–20), 2207-2223. https://doi.org/10.1080/00103629209368735

Simard, R. R., & Zizka, J. (1994). Evaluating plant available potassium with strontium citrate. *Communications in Soil Science and Plant Analysis*, *25*(9–10), 1779–1789. https://doi.org/10.1080/00103629409369152

Simard, R. R., Zizka, J., & Tran, T. S. (1991). Strontium chloride-citric acid extraction evaluated as a soil-testing procedure for phosphorus. *Soil Science Society of America Journal*, *55*(2), 414–421. https://doi.org/10.2136/sssaj1991.03615995005500020021x

Soltanpour, P. N., & Schwab, A. P. (1977). A new soil test for simultaneous extraction of macro- and micro-nutrients in alkaline soils. *Communications in Soil Science and Plant Analysis*, *8*(3), 195–207. https://doi.org/10.1080/00103627709366714

Ståhlberg, S. (1979). A simple turbidimetric method for determination of exchangeable soil potassium. *Communications in Soil Science and Plant Analysis*, *10*(10), 1345–1353. https://doi.org/10.1080/00103627909366988

Sukaton, A. R., Siswoyo, S., & Piluharto, B. (2017). *Optimisation of extractant and extraction time on portable extractor potentiometric method for determining phosphate in soil*. https://jurnal.unej.ac.id/index.php/prosiding/article/view/5255/4009

van Lierop, W. (1986). Sol nitrate determination using the kelowna multiple element extractant. *Communications in Soil Science and Plant Analysis*, *17*(12), 1311–1329. https://doi.org/10.1080/00103628609367792

van Lierop, W. (1988). Determination of available phosphorus in acid and calcareous soils with the kelowna multiple-element extractant. *Soil Science*, *146*(4), 284-291. https://doi.org/10.1097/00010694-198810000-00009

van Lierop, W., & Gough, N. A. (1989). Extraction of potassium and sodium from acid and calcareous soils with the kelowna multiple element extractant. *Canadian Journal of Soil Science*, *69*(2), 235–242. https://doi.org/10.4141/cjss89-024

Vance, C. P., Uhde-Stone, C., & Allan, D. L. (2003). Phosphorus acquisition and use: Critical adaptations by plants for securing a nonrenewable resource. *New Phytologist*, *157*(3), 423–447. https://doi.org/10.1046/j.1469-8137.2003.00695.x

Vendrell, P. F., & Zupancic, J. (1990). Determination of soil nitrate by transnitration of salicylic acid. *Communications in Soil Science and Plant Analysis*, *21*(13-16), 1705–1713. https://doi.org/10.1080/00103629009368334

Waghwani, B., Balpande, S., & Kalambe, J. (2019). Development of microfluidic paper based analytical device for detection of phosphate in water. *International Journal of Innovative Technology and Exploring Engineering*, *8*(6S), 592-595.

Wang, S., Lin, K., Chen, N., Yuan, D., & Ma, J. (2016). Automated determination of nitrate plus nitrite in aqueous samples with flow injection analysis using vanadium (III) chloride as reductant. *Talanta*, *146*, 744–748. https://doi.org/10.1016/j.talanta.2015.06.031

Wang, Y., & Wu, W.-H. (2013). Potassium transport and signaling in higher plants. *Annual Review of Plant Biology*, *64*, 451–476. https://doi.org/10.1146/annurev-arplant-050312-120153

Whittles, C. L., & Little, R. C. (1950). A colorimetric method for the determination of potassium and its application to the analysis of soil extracts. *Journal of the Science of Food and Agriculture*, *1*(11), 323–326. https://doi.org/10.1002/jsfa.2740011103

Pertanika J. Trop. Agric. Sci. 47 (4): 1429 - 1444 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Growth Response and Gene Expression Analysis of Chili Pepper (*Capsicum annuum* L.) Plant Dehydrin Against Salt Stress and Drought *In vitro*

**Elly Syafriani*, Widhi Dyah Sawitri and Ersa Nur Syafia**

*Faculty of Agriculture, Universitas Gadjah Mada, Jl. Flora, Bulaksumur, Caturtunggal, Sleman, 55281, Yogyakarta, Indonesia*

## ABSTRACT

The need for plants resistant to abiotic stress now and in the future is predicted to be very high. It is related to extreme climate change and converting agricultural land into residential and industrial land. As Indonesia's national strategic commodity, chili peppers require special attention when assembling chili peppers resistant to salinity and drought stress. New varieties of chili pepper plants resistant to saline and drought can be obtained through unconventional breeding (overexpression of the dehydrin gene). As a first step in assembling saline and drought-resistant chili plants, growth response and dehydrin gene expression tests were carried out from explants of chili pepper plants of the TM999 variety in vitro on salt and drought treatment media. This study aims to obtain information on the initial response to the growth and expression of the dehydrin gene from chili pepper plants of the TM999 variety before further research is carried out to increase the expression of the dehydrin gene through a molecular approach. The method used in this study is a complete randomized design with two treatments: Sodium chloride (NaCl) and polyethylene glycol (PEG-6000). The results obtained in this study showed that chili pepper varieties TM999 were more tolerant of drought stress than salinity based on several growth response data in both treatments. The analysis of dehydrin gene expression in both treatments showed that the gene expression was strongly influenced by the two strokes given. NaCl and PEG-6000 treatments increased the dehydrin gene expression of chili pepper plants grown *in vitro*.

*Keywords*: Chili pepper, dehydrin, drought stress, morpho-physiology, salt stress

ARTICLE INFO

*Article history:*
Received: 12 January 2024
Accepted: 29 February 2024
Published: 29 November 2024

DOI: https://doi.org/10.47836/pjtas.47.4.22

*E-mail addresses*:
ellysyafriani@ugm.ac.id (Elly Syafriani)
widhi.d.s@ugm.ac.id (Widhi Dyah Sawitri)
ersyafiaa@mail.ugm.ac.id (Ersa Nur Syafia)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

## INTRODUCTION

Chili pepper plants, as a national strategic commodity with an increasing demand every year in Indonesia, often cannot be met by the amount of national chili production, which is relatively low compared to the potential yield. The scarcity of chili peppers and the sudden high price are often found yearly, especially when extreme abiotic stress exists. This condition is exacerbated because agricultural lands have narrowed due to land conversion for residential and industrial needs. Suboptimal land use can be a promising alternative to overcome these problems. However, suboptimal land is known to have several limiting factors for plant growth, including poor nutrients, lack of water, and salinity (Sirappa & Titahena, 2014). Therefore, saline- and drought-resistant chili pepper plants are needed for suboptimal land quickly through unconventional plant breeding (overexpression of dehydrin gene by gene editing method).

Dehydrin is known as subgroup D-11 of the late embryogenesis abundant (LEA) protein that was first identified as expressed during seed maturation and desiccation and was found to be also expressed in vegetative tissues in response to abscisic acid (ABA), drought, salinity, low temperature, and heavy metals (Yuxiu et al., 2007). The utilization of the dehydrin gene to increase abiotic resistance has been proven by several studies (Brini et al., 2007; Meng et al., 2021; Puhakainen et al., 2004). However, experiments on overexpression of the dehydrin gene in chili pepper plants have not been widely reported. Information or knowledge of the dehydrin (DHN) gene in chili pepper plants is still little studied (Jing et al., 2016). Therefore, the novelty of this research will be of considerable value because it will add information related to the expression of the dehydrin gene from chili pepper plants.

The initial stage can be testing the resistance of sampled chili pepper plants (TM999 varieties) under salt stress and drought conditions *in vitro* before the dehydrin gene is modified through gene editing to obtain new varieties of chili pepper plants resistant to salt stress and drought. The aim is to obtain preliminary data on how much the increase in dehydrin gene expression is produced from chili pepper plants when treated with salt stress and drought. The data will then be useful as a baseline or comparative data on the increase in the amount of dehydrin gene expression from sample plants that have received gene modification to prove whether it is true that gene editing can increase the expression (overexpression) of the dehydrin gene of sample plants grown on salt stress and drought treatment media. Therefore, this study aims to study the growth response and expression of the dehydrin gene of chili pepper plants of the TM999 variety *in vitro* with various salt stress (NaCl) and drought stress (PEG-6000) concentration levels.

## MATERIALS AND METHODS

This study was conducted at Plant Tissue Culture and Agrotropica Learning Center (AGLC), Universitas Gadjah Mada,

Daerah Istimewa (D.I.) Yogyakarta, from August to October 2023. The experimental design used in this study was a complete randomized design (CRD). The treatment consists of two factors: Salt stress and drought stress. The chili pepper plant variety used is TM999 (Menteri Pertanian Republik Indonesia, 2005), considering that the variety is most widely planted by farmers in the D.I. Yogyakarta area and has a tolerant nature in lowland planting areas. The salt treatment level in Murashige and Skoog medium with NaCl (MS-NaCl media) consisted of 0, 20, 40, 60, 80, and 100 mM. Meanwhile, the drought treatment level (MS-PEG-6000 media) consisted of 0, 0.5, 1, 2, 4, and 6%. Each level of treatment was repeated as many as 10 repetitions (10 culture bottles), and every culture bottle consisted of 3 plants. So, the total plants used for both treatments were 360 plants.

**Planting**

Explant planting was carried out by preparing treatment media and sterilizing explants for salt stress treatment media consisting of MS media plus NaCl with a composition per liter of media, namely: 4.43 g MS (Phygenera, Germany), 30 g sucrose (Gulaku Murni, Indonesia), 7 g agar (Swallow Globe Brand, Indonesia), and NaCl (Sigma-Aldrich, USA) for the concentration treatments of 100 ml (20 mM), 200 ml (40 mM), 300 ml (60 mM), 400 ml (mM), and 500 ml (100 mM), respectively. In comparison, the drought stress treatment media consists of MS media plus PEG. The composition of 1 L of media (MS-PEG) consisted of 4.43 g MS (Phygenera, Germany), 30 g sucrose (Gulaku Murni, Indonesia), 7 g agar (Swallow Globe Brand, Indonesia), and PEG-6000 (Merck-Germany) for the treatments of 5 g (0.5%), 10 g (1%), 20 g (2%), 40 g (4%), and 60 g (60%), respectively. The explant sterilization procedure was as follows: chili pepper seeds of the TM999 variety were soaked with sterile aqueous for 30 min to help with imbibition; then, in laminar air flow, seeds were soaked with 70% alcohol (Rachma Sari, Indonesia) for 30 s; then the alcohol was removed and continued by soaking at 20% Clorox (Bayclin, Indonesia) for 30 min while shaking on the shaker. Next, the seeds are soaked in sterile aqueous three times, each for 3 min; The seeds were drained and placed on a Petri dish (filled with water) to ensure the seeds did not dry out during culture. After sterilizing the seeds (explants), they were planted in each treatment medium.

**Parameters Observed**

***Growth Parameters***

***Plant Height.*** Plant height was measured six times during the study: 7 days after planting (1 week after planting [WAP]), 2, 3, 4, 5, and 6 WAP. Measurements were made by measuring from the stem's base to the plant's growing point.

***Plant Leaves Number.*** The number of true leaves was calculated by the time the plant was 6 WAP.

***Plant Roots Length and Number.*** The primary root was measured to obtain the root length data by the time the plant was 6 WAP. For root number, the primary and lateral roots were calculated by the time the plant was 6 WAP.

***Plant Stem Diameter.*** The diameter of the stem was measured at 2-3 cm from the base of the stem using a caliper when the plant was 6 WAP old.

***Physiological Parameters***

Physiological observations were made by measuring the diameter and length of stomata and calculating the density or number of stomata. Stomata morphophysiology was observed using the stomata printing method (Sari & Harlita, 2018). The underside of the leaves was coated with transparent nail varnish. The dried layer of nail varnish was peeled off using sellotape and then stuck onto a glass object. The stomatal openings were observed under a microscope using an ocular micrometer at 10× magnification. The ocular micrometer was calibrated with the objective lens at 40× magnification.

***Dehydrin Gene Expression Analysis.***

The mRNA from the dehydrin gene was isolated using the Total RNA Mini Kit (Plant) w/DNase Set (RPD100, Geneaid Biotech Ltd., Taiwan). The RNA isolation results were then quantified by NanoDrop (Biochrom, United Kingdom). The cDNA synthesis was carried out with the help of Toyobo FSQ-101 ReverTra Ace® qPCR RT kit (Japan). The synthesis of cDNA began with a dilution of extracted RNA according to the RNA quantification value: the formula used was 500 ng/known RNA concentration (ng/µl). Next, a cDNA synthesis cocktail was made with the following composition: 5× RT Buffer (2 µl), primer mix (0.5 µl), enzyme mix (0.5 µl), RNA (depending on the results of quantification calculations needed for dilution), and nuclease-free water adjusted the amount of RNA used with the final volume of the cocktail, which was 10 µl. Furthermore, incubation was carried out with the help of a polymerase chain reaction (PCR) machine with the following reaction: temperature 37ºC for 1 hr, 98ºC for 5 min, and the final temperature of the reaction was 10ºC (∞). After cDNA was successfully synthesized, the next step was the PCR process using a real-time PCR machine (QuantStudio™ 3, Thermo Fisher Scientific, USA). Before PCR, PCR cocktails are prepared using Bioline BIO-94005 SensiFAST SYBR Lo-ROX Kit (Thermo Fisher Scientific, USA) with cocktail composition: 2× SensiFAST SYBR Lo-ROX (10 µl), 10 µM forward primer (0.8 µl), 10 µM reverse primer (0.8 µl), cDNA (1 µl), $H_2O$ (7.4 µl), so that the total final volume was (20 µl). Each sample was to be amplified with both specific primers and control primers and was repeated 3×. The specific primer sequences to amplify the dehydrin gene used (Chen et al., 2015) are (1) forward primer (qCaDHN1-F) 5'-AGTGATCATTCTTTGCTTTTTATTC-3', and (2) reverse primer (qCaDHN1-R) 5'-TTAACTTTCTCACCAAACTCAGA-3'. While the primer control sequence

used (Chen et al., 2015) was (1) forward primer (qCaUbi3-F) 5'-TGTCCATCTGCTCTCTCTCTTG-3', and (2) reverse primer (qCaUbi3-R) 5'CACCCCAAGCACAATAAGAC-3'. The PCR programs used were denaturation for 5 s (95ºC), annealing for 10 s (54 ºC), and extension for 5 s (72ºC). The total number of cycles used was 40. After the PCR results were obtained, the data was analyzed using the Livak method (Schmittgen & Livak, 2008).

### *Data Analysis*

The obtained data were analyzed using analysis of variance with a significance level of 5%, followed by a post hoc honestly significant difference (HSD) Tukey's test to find the significant differences between treatment groups.

## RESULTS AND DISCUSSION

### Growth Response of Chili Pepper Plants in Salt and Drought Treatment Media

The growth response of chili pepper plants in salt stress and drought treatment media (Figures 1A-1B) showed that salt stress treatment had a more significant effect on plant height growth than drought stress treatment in each observation week. The trend graph in both treatments shows a decrease in plant height in accordance with the higher concentrations of NaCl and PEG. The data in Figure 1A also showed that the decrease in plant height at salt treatment (NaCl) with high concentration (100 mM) could reach about twice the height of control plants (0 mM). Unlike the data in Figure 1B, although all plants had reduced PEG concentration levels compared to controls, the decrease was not as significant as that of salt treatment. The height difference between the control and highest PEG concentration plants (6%) was only about 0.56 cm apart. According to Xiao and Zhou (2023), soil salinization is one of the most detrimental environmental stresses, severely restricting plant growth and development and threatening agricultural production worldwide. So, it is not surprising that from the two choking comparisons given in this study, a more significant growth reduction response was shown in plantlets in salt stress media than plantlets in PEG media. According to van Zelm et al. (2020), salt stress causes inhibition of plant growth, abnormal development, and metabolic disorders. Generally, plants that experience salt stress will inhibit water absorption in their body tissues due to the viscosity of tissue fluid. According to Ludwiczak et al. (2021) as well as Yang and Guo (2018), the adverse effects of high salinity in plants are (1) osmotic stress as sodium (Na) accumulation in the soil and (2) ionic stress. Furthermore, osmotic stress is caused by hyperosmotic soil fluid disrupting plant cell pressure. In contrast, ionic stress is characterized by disrupting the sodium or potassium balance in cells, thus disrupting most metabolic and physiological processes (Zhang et al., 2018).

Osmotic stress is also found in plants that experience drought stress. Rao et al. (2006) stated that drought causes plants to experience an increase in osmotic pressure

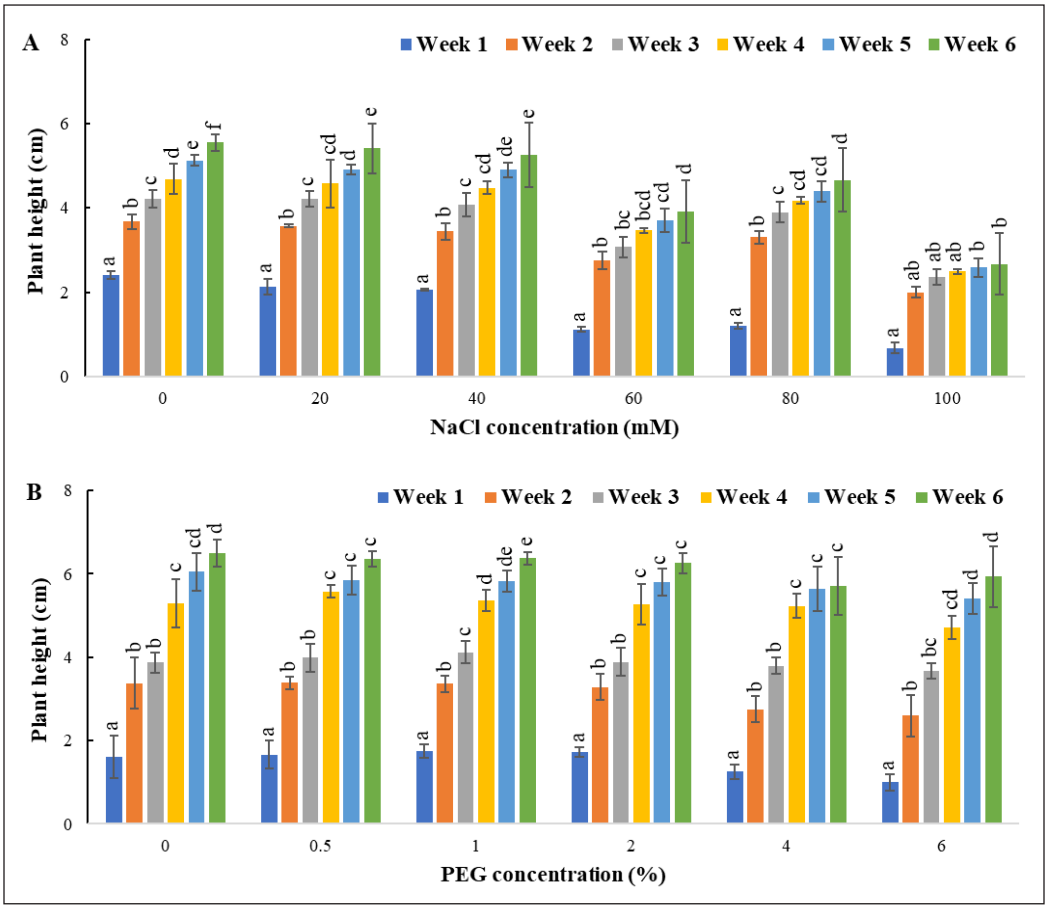



*Figure 1*. The average chili pepper plants' heights under (A) sodium chloride (NaCl) and (B) polyethylene glycol (PEG) exposure using various levels of concentration, respectively
*Note*. Bars showing the same letters at the same concentration level are insignificantly different based on honestly significant difference Tukey's test at α = 5%

and a decrease in cell turgor pressure. Therefore, the response of sample plants in this study, both in salt and PEG stress media, showed a decrease in plant height, which was predicted to occur due to osmotic stress events experienced by these plants. Cushman (2001) supported this, stating that salinity and drought cause osmotic stress inhibiting plant growth. Osmotic pressure or stress is a growth inhibition in the form of a decrease in external water potential as a physiological response of plants to salinity and drought. Lowering the external water potential triggers several major events in plant tissues. For example, at the macroscopic level, osmotic stress inhibits the increase in cells due to decreased turgor pressure (Zhao et al., 2020), so plant cell walls wrinkle and sag (Ma et al., 2020). Poorter et al. (2012) report that when plants experience severe drought stress, they can decrease their biomass by up to 50% compared to control crops, accompanied by an increase in root mass fraction, which

can largely be attributed to a decrease in stem growth. In the face of osmotic stress, plants maintain root growth and reduce shoot growth, especially in the early stages of growth (Ma et al., 2020).

Further plant growth data, namely the number of leaves (Table 1), showed a significant difference between the number of leaves of control plants and sample plants in both treatments (salt and drought), especially in the treatment with the highest concentration. In salt treatment, the number of leaves between the control plant and the highest NaCl concentration treatment plant (100 mM) was two leaves, while the difference in the number of leaves of the control plant with the other four NaCl concentration treatments was only one leaf. The results are also the same in plants with PEG treatment. According to Farooq et al. (2009), drought affects plant growth and development with substantial reductions in the speed of growth and biomass accumulation. With this influence, it is not surprising that the number of leaves of the treatment plant is not more than that of the control plant because of the difference in growth speed, as mentioned earlier. The main consequences of drought in plants are reduced cell division and expansion speed, leaf size, stem elongation, and stem proliferation, disrupting stomatal oscillations, linking plant nutrients and water with reduced plant productivity, and efficient water use. In another mechanism, it is known that plants respond to drought conditions by reducing leaves through leaf shedding to reduce excess transpiration (Seleiman et al., 2021). Unfortunately, this study did not observe the number of fallen leaves. It is related to the large number of plants used, making it difficult to make observations simultaneously as observations of other variables. However, the variable number of leaves significantly differs between control and treatment plants, which has adequately illustrated the plant's response to leaf growth as an influence of both treatments.

Observations on the length and number of plant roots in both treatment media showed that both treatments significantly

Table 1
*The number of leaves of chili pepper plant var. TM999 in salt and drought treatments at 6 WAP*

| NaCl treatment (mM) | Leaf number | | PEG treatment (%) | Leaf number | |
|---|---|---|---|---|---|
| 0 | 5.78 ± 0.83 | a | 0 | 6.22 ± 1.20 | a |
| 20 | 5.11 ± 0.60 | ab | 0.5 | 5.22 ± 0.83 | ab |
| 40 | 4.89 ± 0.60 | ab | 1 | 5.11 ± 0.76 | ab |
| 60 | 4.78 ± 0.83 | ab | 2 | 4.78 ± 0.67 | ab |
| 80 | 4.56 ± 0.88 | ab | 4 | 5.22 ± 0.67 | b |
| 100 | 3.67 ± 2.12 | b | 6 | 4.67 ± 0.71 | b |
| CV (%) | 19.78 | | CV (%) | 16.44 | |

*Note*. Means in the same column followed by the different letters are significantly different at 0.005 probability level; WAP = Weeks after planting; NaCl = Sodium chloride; PEG = Polyethylene glycol; CV = Coefficient of variation

affected the size and number of roots (Table 2). Salt and PEG stress treatments have an impact in the form of increasing root length compared to controls. Different results were found in the variable number of roots, where in salt treatment, the number of roots tended to decrease compared to controls. In PEG treatment, the effect tends to increase the number of roots.

Plants respond to salt stress and drought by increasing root length as self-defense. In growth and development, plants depend on water availability for the metabolic processes of cells and bodies. In nature, in conditions of salt stress and drought, plants make modifications by increasing root length, which is useful as an extension tool in obtaining water at a certain depth of soil or outside the header area. This phenomenon also applies to the results obtained in this study. The root length of the treatment plant exceeds the root length of the control plant to assist the plant in obtaining water at a given depth and area of the medium. As the organ that first senses water shortage, roots feel stress immediately after exposure to drought stress and produce a specific response to the drought stress. Physiologically, drought stress causes changes in some metabolic pathways of plants. The allocation of photosynthates in the roots and rhizosphere is inhibited in severe drought conditions. It reduces the absorption of water and nutrients by the roots, stunts growth, and ultimately affects biomass and plant yield accumulation. In drought stress conditions, a well-developed root system architecture can improve the quality of plant drought

Table 2
*The length and number of roots of chili pepper plant var. TM999 under salt and drought treatment at 6 WAP*

| NaCl treatment (mM) | Root length (cm) | | PEG treatment (%) | Root length (cm) | |
|---|---|---|---|---|---|
| 0 | 6.23 | b | 0 | 8.43 ± 1.87 | b |
| 20 | 9.50 | ab | 0.5 | 5.08 ± 0.75 | c |
| 40 | 9.00 | ab | 1 | 7.39 ± 3.28 | bc |
| 60 | 9.73 | ab | 2 | 8.07 ± 2.32 | bc |
| 80 | 7.94 | ab | 4 | 6.74 ± 2.42 | bc |
| 100 | 10.21 | a | 6 | 13.81 ± 4.24 | a |
| CV (%) | 16.00 | | CV (%) | 16.03 | |
| NaCl treatment (mM) | Number of roots | | PEG treatment (%) | Number of roots | |
| 0 | 9.56 ± 2.79 | a | 0 | 4.67 ± 1.00 | b |
| 20 | 5.22 ± 1.48 | b | 0.5 | 4.89 ± 1.05 | b |
| 40 | 5.00 ± 1.41 | b | 1 | 5.22 ± 1.64 | b |
| 60 | 2.78 ± 0.67 | c | 2 | 6.89 ± 2.03 | ab |
| 80 | 2.56 ± 0.52 | c | 4 | 9.33 ± 3.43 | a |
| 100 | 2.33 ± 0.87 | c | 6 | 6.44 ± 1.67 | ab |
| CV (%) | 14.98 | | CV (%) | 17.39 | |

*Note*. Means in the same column followed by the different letters are significantly different at 0.005 probability level; WAP = Weeks after planting; NaCl = Sodium chloride; PEG = Polyethylene glycol; CV = Coefficient of variation

tolerance and the utilization of limited resources (water and nutrients). Optimized root system architecture can strengthen the properties of plants facing drought stress, including the length and number of roots, to increase the absorption of deeper water sources, ultimately increasing drought tolerance (Kang et al., 2022).

The difference in root count response in salt and PEG treatment plants (Table 2) shows that chili pepper plants of the TM999 variety have different mechanisms in dealing with the two types of stress. In salt stress, plants tend to reduce lateral root growth, although lateral roots are known to be very important for water absorption. It is well known that the effect of salt stress on plants can result in osmotic pressure that inhibits cell division and elongation, so it will automatically also inhibit lateral root growth and reduce their number. In contrast to the number of roots in drought-treated plants that tend to increase compared to control plants, these results show facts that contradict existing theories. In many cases, lateral root growth will be limited to water shortage conditions (Durand et al., 2016). The results of this study suggest that drought stress can increase the number of lateral roots in certain plant species (in this case, chili peppers). In other words, the TM999 chili pepper plant variety used in this study is predicted to have genetic factors that can help its root system deal with drought stress by increasing the number of lateral roots to increase water and nutrient absorption. This phenomenon was also found in another study by Chun et al. (2021) on soybean plants.

Furthermore, this study also found a noticeable influence exerted by both treatments on stem diameter (Table 3). The higher the treatment concentration given, the smaller the diameter of the plant stem. As previously stated, salinity and drought treatment will reduce the weight of plant mass, including inhibition of stem growth through decreasing the number and size of cells.

Stomatal morphophysiological data (Table 4) showed that salt stress treatment had a significant effect on stomatal length

Table 3
*The stem diameter of the chili pepper plant var. TM999 under salt and drought treatment at 6 WAP*

| NaCl treatment (mM) | Stem diameter (cm) | | PEG treatment (%) | Stem diameter (cm) | |
|---|---|---|---|---|---|
| 0 | 0.83 ± 0.05 | a | 0 | 0.81 ± 0.07 | a |
| 20 | 0.72 ± 0.67 | b | 0.5 | 0.83 ± 0.15 | a |
| 40 | 0.68 ± 0.04 | b | 1 | 0.79 ± 0.12 | a |
| 60 | 0.57 ± 0.10 | c | 2 | 0.71 ± 0.12 | a |
| 80 | 0.54 ± 0.10 | c | 4 | 0.69 ± 0.09 | a |
| 100 | 0.42 ± 0.08 | d | 6 | 0.49 ± 0.18 | b |
| CV (%) | 11.92 | | CV (%) | 17.75 | |

*Note*. Means in the same column followed by the different letters are significantly different at 0.005 probability level; WAP = Weeks after planting; NaCl = Sodium chloride; PEG = Polyethylene glycol; CV = Coefficient of variation

and had no noticeable impact on stomatal diameter (width) and stomatal density (number). In contrast to drought stress treatment plants, a noticeable influence is not only shown on the length of the stomata but also the diameter (width) of the stomata. Meanwhile, the density (number) of stomata does not influence the provision of drought stress treatment. In order, the three treatment plants with the highest to lowest stomata length in NaCl treatment were 20, 0 (control), and 100 mM concentration, with their respective lengths of 13.97, 9.7, and 5.72 µm. These results indicate that NaCl treatment at a certain concentration (20 mM) can increase the stomata length of chili pepper plants var. TM999. Meanwhile, more than 20 mM NaCl concentrations can decrease stomatal length compared to control plants. Not much different from the size of the average diameter of the stomata of PEG treatment plants, consistently all PEG concentrations tried had an effect in the form of a decrease in stomatal length in line with the higher PEG concentrations used. Based on available data, the length of stomata in PEG treatment plants is quite variable, where the longest stomata are 11.07 µm (0% PEG), and the shortest stomata are found in PEG treatment concentrations of 2% (6.53 µm). However, the stomata diameter length of certain PEG concentration treatment plants (0.5 and 6%) has a longer stomata length when compared to the stomata length of other concentration treatment plants (PEG >0.5%<6%).

Varying yields were also found in the stomata diameter of PEG treatment plants. If

Table 4
*The observation of stomata of chili pepper plant var. TM999 under salt and drought treatment at 6 WAP*

| NaCl treatment (mM) | Stomata diameter (µm) | | Stomata length (µm) | | Stomata density (n/mm$^2$) | |
|---|---|---|---|---|---|---|
| 0 | 12.07 | a | 9.70 | b | 367.00 | a |
| 20 | 14.70 | a | 13.97 | a | 223.50 | a |
| 40 | 12.69 | a | 4.73 | c | 239.00 | a |
| 60 | 14.80 | a | 4.45 | c | 202.50 | a |
| 80 | 11.96 | a | 4.31 | c | 106.50 | a |
| 100 | 12.42 | a | 5.72 | c | 163.50 | a |
| CV (%) | 17.10 | | 20.47 | | 8.13 | |
| PEG treatment (%) | Stomata diameter (µm) | | Stomata length (µm) | | Stomata density (n/mm$^2$) | |
| 0 | 14.10 | a | 11.07 | a | 259.00 | a |
| 0.5 | 15.32 | a | 9.36 | a | 309.00 | a |
| 1 | 9.66 | b | 7.11 | b | 87.50 | a |
| 2 | 13.33 | a | 6.53 | b | 312.00 | a |
| 4 | 10.03 | b | 6.91 | b | 322.00 | a |
| 6 | 9.61 | b | 9.61 | a | 155.50 | a |
| CV (%) | 14.78 | | 18.07 | | 7.03 | |

*Note*. Means in the same column followed by the different letters are significantly different at 0.005 probability level; WAP = Weeks after planting; NaCl = Sodium chloride; PEG = Polyethylene glycol; CV = Coefficient of variation

the stomata diameter range of salt treatment plants is relatively narrow, which is between 11.96 and 14.80, then the stomata diameter range of PEG treatment plants is quite wide, which is 9.61 to 15.32. Variations in stomatal diameter data in PEG treatment illustrate that plants respond in the form of a decrease in stomatal width or diameter compared to controls. The reduction in stomata size (diameter and length) in treatment plants compared to stomata size in control plants indicates that plants respond to salt stress and drought through decreased physiological activity, namely transpiration. The size of stomata that are smaller than their normal size is believed to avoid water loss quickly (Li et al., 2017). However, the size of the stomata is affected by the treatment, not by the number or density of stomata. Stomata are needed plants for gas exchange. Generally, the amount or density is greatly influenced by the amount of carbon dioxide ($CO_2$) available in the plant-growing environment. If enough $CO_2$ is available, the number of stomata is not greater than when there is less $CO_2$ available in the air (Xu et al., 2016). Therefore, this study's salt and drought treatment did not affect stomatal density.

The salt stress and drought treatment results in this study showed that chili pepper plants of the TM999 variety gave a dominant morphological growth response (Figure 2) affected by both treatments rather than their physiological growth response (stomata morphophysiology). It can happen with the suspicion that organ modifications carried out by chili pepper plants of the TM999 variety in the form of an increase in the length and number of roots followed

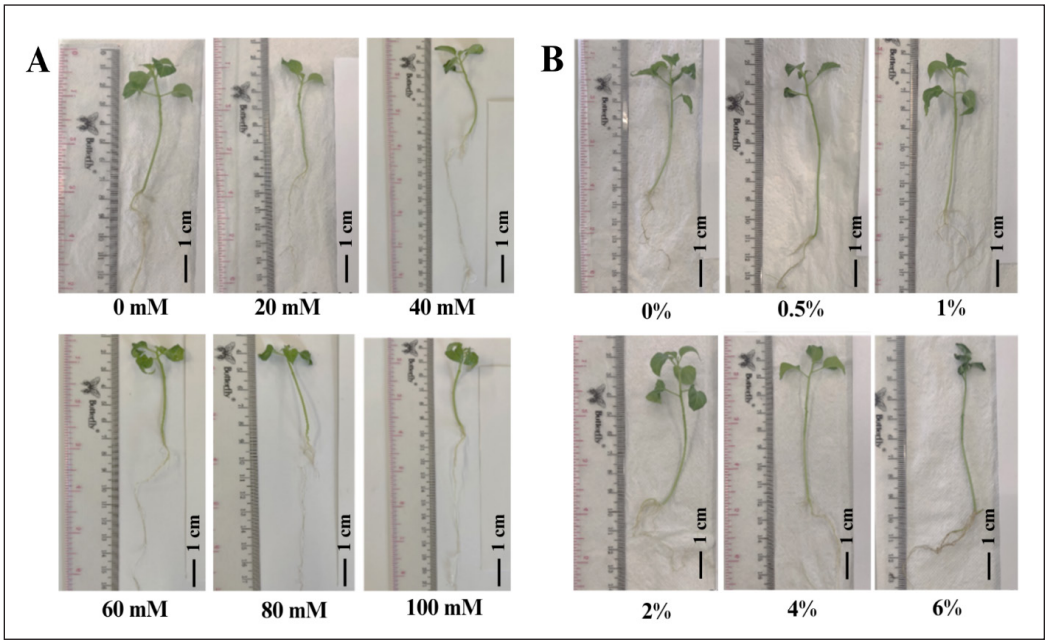



*Figure 2*. Chili pepper plants' morphological documentation under different abiotic stress conditions at 6 weeks after planting: (A) saline stress; (B) drought stress

by a reduction in plant height, number of leaves, stem diameter, and stomata size due to the two treatments given, turned out to be able to help plants in carrying out their physiological activities to remain in normal conditions.

Growth is an irreversible volume, size, or weight increase, including the phases of cell division, cell elongation, and cell differentiation, in which cell division and enlargement are affected by drought stress conditions due to disruption of enzyme activity, turgor loss, and decreased energy supply (Farooq et al., 2009). In conditions of salt stress and drought, there will be a decrease in dry matter accumulation in all plant organs. However, different organs show different levels of decline according to the data obtained in this study. Other studies also add information that drought stress causes a decrease in leaf area and leaf count due to turgor loss (Farooq et al., 2010).

**Dehydrin Gene Expression Analysis**

The tolerant nature of chili pepper plants of the TM999 variety against salt stress and drought in this study cannot be separated from its ability to increase the production of dehydrin compounds. Dehydrin is an intrinsically irregular protein belonging to one of the late embryogenesis abundant (LEA) family of genes. LEA is a protein that plants express as one of the molecular mechanisms in response to abiotic stress. *In vitro* evidence shows that dehydrin is involved in the protection of membranes, proteins, and DNA from abiotic stress. So, dehydrin is considered an abiotic stress-protective protein with multiple roles (Smith & Graether, 2022).

The dehydrin gene expression analysis results in plants from both treatment types showed a significant increase in expression compared to control plants. Figure 3A shows the highest to lowest increase in dehydrin gene expression in NaCl treatment with concentrations of 20, 100, 60, and 40 mM. While in NaCl treatment concentration of 80 mM, there was a decrease in dehydrin gene expression. It is not yet known why a concentration of 80 mM NaCl can decrease the expression of the dehydrin gene of red pepper plants var. TM999 in this study, compared to other concentration levels, tends to increase the expression of the gene. However, this is thought to occur because the increase in dehydrin gene expression is largely determined by the control of interactions between genes and other molecules that are influenced by the concentration of the abiotic stress inducer given. Therefore, no linear pattern was found between increased NaCl concentrations and dehydrin gene expression. A similar result to this study has been found in the research of Alharby et al. (2016), which studied mRNA expression of *superoxide dismutase* (*SOD*) and *glutathione peroxidase* (*GPX*) genes in tomatoes under stress NaCl and/or zinc oxide (ZnO) nanoparticles. Based on Alharby et al. (2016) research, which investigated the effect of nanoparticles of zinc oxide (NPs-ZnO) on the expression mRNA levels of *SOD* and *GPX* genes under salinity stress and confirmed that

a decrease in mRNA expression of *SOD* and *GPX* genes occurred during exposure to NaCl. In contradictive, Alharby et al. (2016) research has also found that a low and/or appropriate dose of NPs-ZnO has a positive response on plant metabolism to increase the expression of mRNA levels of the *SOD* and *GPX* genes in tomatoes under salinity. Another assumption was that the increases in the mRNA levels of *SOD* and *GPX* genes could be a result of increased stability of transcribed mRNAs. Further research was needed in this study to reveal the other molecules that play an important role in affecting the decreasing and increasing of DHN1 mRNA expression levels under salinity. These other molecules could be predicted from the MS medium content used in this study.

In contrast to the results obtained in the PEG treatment (Figure 3B), a linear pattern was found between an increase in the concentration of PEG used and an increase in the expression of the dehydrin gene. The highest to lowest increases in dehydrin gene expression in PEG treatment were 6, 2, 1, and 4%, respectively. At a PEG concentration of 0.5%, there was a decrease in dehydrin gene expression. The lowest increase in dehydrin gene expression at a PEG concentration level of 4% further strengthens previous predictions that increased dehydrin gene expression is largely determined by control of interactions between genes and other molecules. Although higher eukaryotes still respond to environmental signals by regulating their genes, there is an additional layer of regulation resulting from cell-to-cell interactions that regulate the development of an organism. In the end, although there was a variation in the increase and decrease in dehydrin gene expression between concentration treatment levels of both NaCl

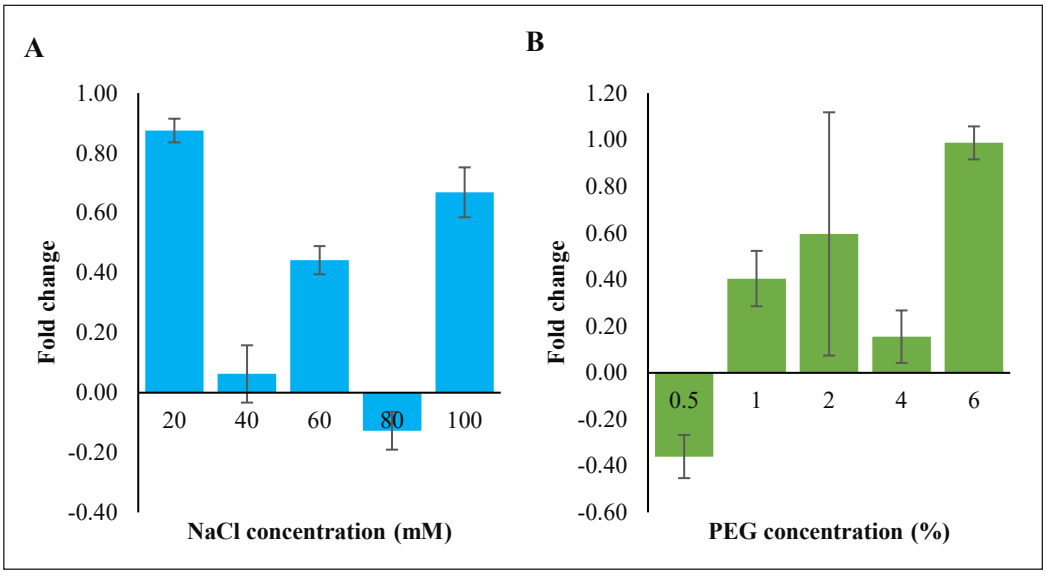



*Figure 3*. Dehydrin gene expression analysis under different abiotic stress conditions at 6 weeks after planting: (A) sodium chloride (NaCl) treatment; (B) polyethylene glycol (PEG) treatment

and PEG, it did not change the fact that salt and drought stress applied were able to significantly increase the expression of the dehydrin gene in the treatment plants compared to the respective control plants. The high expression of the dehydrin gene in chili pepper plants of the TM999 variety in both treatments (NaCl and PEG) proved the role of dehydrin compounds in helping to increase plant resistance to salt stress and drought when associated with morphological and physiological growth responses. The average morphological and physiological growth response of treatment plants in this study is classified as able to catch up or be parallel to the growth of control plants, especially in certain observation variables that show no real influence between treatment plants and control plants.

## CONCLUSION

The chili pepper plants of the TM999 variety are tolerant to both types of stress treatments tried, especially drought stress, and have the potential to increase their resistance through molecular approaches or over-expression of dehydrin genes with a gene editing method.

## ACKNOWLEDGMENTS

The authors would like to express their gratitude to the Directorate General of Higher Education, Research, and Technology, Indonesia, which fully funded this research through the Fundamental Research Grant in 2023, assignment letter number 122/E5/PG.02.00.PL/2023.

## REFERENCES

Alharby, H. F., Metwali, E. M. R., Fuller, M. P., & Aldhebiani, A. Y. (2016). The alteration of mRNA expression of SOD and GPX genes, and proteins in tomato (*Lycopersicon esculentum* Mill) under stress of NaCl and/or ZnO nanoparticles. *Saudi Journal of Biological Sciences*, *23*(6), 773–781. https://doi.org/10.1016/j.sjbs.2016.04.012

Brini, F., Hanin, M., Lumbreras, V., Amara, I., Khoudi, H., Hassairi, A., Pagès, M., & Masmoudi, K. (2007). Overexpression of wheat dehydrin DHN-5 enhances tolerance to salt and osmotic stress in *Arabidopsis thaliana*. *Plant Cell Reports*, *26*, 2017–2026. https://doi.org/10.1007/s00299-007-0412-x

Chen, R.-G., Jing, H., Guo, W.-L., Wang, S.-B., Ma, F., Pan, B.-G., & Gong, Z.-H. (2015). Silencing of dehydrin *CaDHN1* diminishes tolerance to multiple abiotic stresses in *Capsicum annuum* L. *Plant Cell Reports*, *34*, 2189–2200. https://doi.org/10.1007/s00299-015-1862-1

Chun, H. C., Lee, S., Choi, Y. D., Gong, D. H., & Jung, K. Y. (2021). Effects of drought stress on root morphology and spatial distribution of soybean and adzuki bean. *Journal of Integrative Agriculture*, *20*(10), 2639–2651. https://doi.org/10.1016/S2095-3119(20)63560-2

Cushman, J. C. (2001). Osmoregulation in plants: Implications for agriculture. *American Zoologist*, *41*(4), 758–769. https://doi.org/10.1668/0003-1569(2001)041[0758:OIPIFA]2.0.CO;2

Durand, M., Porcheron, B., Hennion, N., Maurousset, L., Lemoine, R., & Pourtau, N. (2016). Water deficit enhances C export to the roots in *Arabidopsis thaliana* plants with contribution of sucrose transporters in both shoot and roots. *Plant Physiology*, *170*(3), 1460–1479. https://doi.org/10.1104/pp.15.01926

Farooq, M., Basra, S. M. A., Wahid, A., Ahmad, N., & Saleem, B. A. (2009). Improving the drought

tolerance in rice (*Oryza sativa* L.) by exogenous application of salicylic acid. *Journal of Agronomy and Crop Science*, *195*(4), 237–246. https://doi.org/10.1111/j.1439-037X.2009.00365.x

Farooq, M., Kobayashi, N., Ito, O., Wahid, A., & Serraj, R. (2010). Broader leaves result in better performance of indica rice under drought stress. *Journal of Plant Physiology*, *167*(13), 1066–1075. https://doi.org/10.1016/j.jplph.2010.03.003

Jing, H., Li, C., Ma, F., Ma, J.-H., Khan, A., Wang, X., Zhao, L.-Y., Gong, Z.-H., & Chen, R.-G. (2016). Genome-wide identification, expression diversication of dehydrin gene family and characterization of *CaDHN*$_3$ in pepper (*Capsicum annuum* L.). *PLOS One*, *11*(8), e0161073. https://doi.org/10.1371/journal.pone.0161073

Kang, J., Peng, Y., & Xu, W. (2022). Crop root responses to drought stress: Molecular mechanisms, nutrient regulations, and interactions with microorganisms in the rhizosphere. *International Journal of Molecular Sciences*, *23*(16), 9310. https://doi.org/10.3390/ijms23169310

Li, Y., Li, H., Li, Y., & Zhang, S. (2017). Improving water-use efficiency by decreasing stomatal conductance and transpiration rate to maintain higher ear photosynthetic rate in drought-resistant wheat. *The Crop Journal*, *5*(3), 231–239. https://doi.org/10.1016/j.cj.2017.01.001

Ludwiczak, A., Osiak, M., Cárdenas-Pérez, S., Lubińska-Mielińska, S., & Piernik, A. (2021). Osmotic stress or ionic composition: Which affects the early growth of crop species more? *Agronomy*, *11*(3), 435. https://doi.org/10.3390/agronomy11030435

Ma, Y., Dias, M. C., & Freitas, H. (2020). Drought and salinity stress responses and microbe-induced tolerance in plants. *Frontiers in Plant Science*, *11*, 591911. https://doi.org/10.3389/fpls.2020.591911

Meng, Y.-C., Zhang, H.-F., Pan, X.-X., Chen, N., Hu, H.-F., ul Haq, S., Khan, A., & Chen, R.-G. (2021). *CaDHN*$_3$, a pepper (*Capsicum annuum* L.) dehydrin gene enhances the tolerance against salt and drought stresses by reducing ROS accumulation. *International Journal of Molecular Sciences*, *22*(6), 3205. https://doi.org/10.3390/ijms22063205

Menteri Pertanian Republik Indonesia. (2005). *Pelepassan cabe keriting hibrida TM 999 sebagai varietas unggul* [Release of hybrid curly pepper TM 999 as a superior variety]. https://benih.pertanian.go.id/storage/VdemE1na7WTqlTyhPTeiIcP8p9BdXp-metaU0sgQ2FiYWkgS2VyaXRpbmcgVE0gOTk5LnBkZg==-.pdf

Poorter, H., Niklas, K. J., Reich, P. B., Oleksyn, J., Poot, P., & Mommer, L. (2012). Biomass allocation to leaves, stems and roots: Meta-analyses of interspecific variation and environmental control. *The New Phytologist*, *193*(1), 30–50. https://doi.org/10.1111/j.1469-8137.2011.03952.x

Puhakainen, T., Hess, M. W., Mäkelä, P., Svensson, J., Heino, P., & Palva, E. T. (2004). Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in *Arabidopsis*. *Plant Molecular Biology*, *54*, 743–753. https://doi.org/10.1023/B:PLAN.0000040903.66496.a4

Rao, K. V. M., Raghavendra, A. S., & Reddy, K. J. (Eds.). (2006). *Physiology and molecular biology of stress tolerance in plants*. Springer. https://doi.org/10.1007/1-4020-4225-6

Sari, D. P., & Harlita. (2018). Preparasi hands free section dengan teknik replika untuk identifikasi stomata [Hand free section preparation through replica technique for stomata identification]. *Proceeding Biology Education Conference: Biology, Science, Enviromental, and Learning*, *15*(1), 660-664.

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative $C_T$

method. *Nature Protocols*, *3*, 1101–1108. https://doi.org/10.1038/nprot.2008.73

Seleiman, M. F., Al-Suhaibani, N., Ali, N., Akmal, M., Alotaibi, M., Refay, Y., Dindaroglu, T., Abdul-Wajid, H. H., & Battaglia, M. L. (2021). Drought stress impacts on plants and different approaches to alleviate its adverse effects. *Plants*, *10*(2), 259. https://doi.org/10.3390/plants10020259

Sirappa, M. P., & Titahena, M. L. J. (2014). Improvement of suboptimal land productivity approach by land and plant management. *Journal of Tropical Soils*, *19*(2), 99–109.

Smith, M. A., & Graether, S. P. (2022). The disordered dehydrin and its role in plant protection: A biochemical perspective. *Biomolecules*, *12*(2), 294. https://doi.org/10.3390/biom12020294

van Zelm, E., Zhang, Y., & Testerink, C. (2020). Salt tolerance mechanisms of plants. *Annual Review of Plant Biology*, *71*, 403–433. https://doi.org/10.1146/annurev-arplant-050718-100005

Xiao, F., & Zhou, H. (2023). Plant salt response: Perception, signaling, and tolerance. *Frontiers in Plant Science*, *13*, 1053699. https://doi.org/10.3389/fpls.2022.1053699

Xu, Z., Jiang, Y., Jia, B., & Zhou, G. (2016). Elevated-$CO_2$ response of stomata and its dependence on environmental factors. *Frontiers in Plant Science*, *7*, 657. https://doi.org/10.3389/fpls.2016.00657

Yang, Y., & Guo, Y. (2018). Elucidating the molecular mechanisms mediating plant salt-stress responses. *The New Phytologist*, *217*(2), 523–539. https://doi.org/10.1111/nph.14920

Yuxiu, Z., Zi, W., & Jin, X. (2007). Molecular mechanism of dehydrin in response to environmental stress in plant. *Progress in Natural Science*, *17*(3), 237–246. https://doi.org/10.1080/10020070612331343254

Zhang, Y., Lv, Y., Jahan, N., Chen, G., Ren, D., & Guo, L. (2018). Sensing of abiotic stress and ionic stress responses in plants. *International Journal of Molecular Sciences*, *19*(11), 3298. https://doi.org/10.3390/ijms19113298

Zhao, C., Zhang, H., Song, C., Zhu, J.-K., & Shabala, S. (2020). Mechanisms of plant responses and adaptation to soil salinity. *The Innovation*, *1*(1), 100017. https://doi.org/10.1016/j.xinn.2020.100017

Pertanika J. Trop. Agric. Sci. 47 (4): 1445 - 1471 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Bioefficacy of Bio-insecticide from *Chromolaena odorata* (L.) R. M. King & H. E. Robins Methanol Extract against Brown Planthopper, *Nilaparvata lugens* (Stål.)

**Nor Ilya Mohd Zaki[1], Norhayu Asib[2], Erwan Shah Shari[3] and Muhammad Saiful Ahmad-Hamdani[1]***

*[1]Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
*[2]Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
*[3]Rice Research Center, Malaysian Agricultural Research and Development Institute, MARDI Seberang Perai, 13200 Kepala Batas, Pulau Pinang, Malaysia*

## ABSTRACT

The rice brown planthopper (BPH), *Nilaparvata lugens* (Stål.), is a highly damaging insect pest to rice crops. The excessive use of synthetic chemicals has resulted in the development of resistance to insecticides and negative consequences for the environment and insect biodiversity. Hence, three common weed species, namely *Ageratum conyzoides*, *Chromolaena odorata*, and *Mallotus paniculatus*, were evaluated on the comparative extraction yield in different solvents, as well as the toxicity potential in the selected methanol extract. Further, the bioactive compounds in *C. odorata* were characterized, and potential bio-insecticide formulations were developed and evaluated on the BPH. Methanol extract displayed higher efficiency, yielding 17.29% compared to only 3.19% in hexane extract. Insecticidal activity evaluation demonstrated that *C. odorata* exhibited the highest toxicity (77.50% at 10,000 ppm), having a median lethal concentration ($LC_{50}$) value of 977 ppm, while *A. conyzoides* (55.20% at 10,000 ppm) and *M. paniculatus* (60.0% at 12,000 ppm) produced $LC_{50}$ values of 6,549 ppm and 21,940 ppm, respectively. Subsequently, a plant-based bio-insecticide was formulated using the crude methanol extract of *C. odorata* as the active ingredient. A mixture of surfactant (Emersense® AM 8025), oil (palm kernel oil ester), and water resulted in a stable macroemulsion called Emersense® AM 8025/palm kernel oil

ARTICLE INFO

*Article history:*
Received: 18 September 2023
Accepted: 14 December 2023
Published: 29 November 2024

DOI: https://doi.org/10.47836/pjtas.47.4.23

*E-mail addresses*:
norilyazaki@gmail.com (Nor Ilya Mohd Zaki)
norhayuasib@upm.edu.my (Norhayu Asib)
erwanss@mardi.gov.my (Erwan Shah Shari)
s_ahmad@upm.edu.my (Muhammad Saiful Ahmad-Hamdani)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

ester/water (EM-PKOE). The formulated macroemulsion displayed enhanced toxicity and efficacy against BPH nymphs, with an $LC_{50}$ value of 220 ppm, outperforming the unformulated crude methanol extract (977 ppm). Chemical composition analysis using gas chromatography-mass spectrometry revealed that *C. odorata* primarily contained sesquiterpenes (24.14%). This study proposes *C. odorata* as a potential bio-insecticide for BPH combatants, necessitating further research on the formulation for eventual commercialization to sustainable BPH control in rice cultivation.

*Keywords*: Bio-insecticide, brown planthopper, crude plant extract, emulsion formulation, gas chromatography-mass spectrometry

## INTRODUCTION

Brown planthopper (BPH) (*Nilaparvata lugens* (Stål).; Hemiptera: Delphacidae) has always been a major threat to rice crops globally, causing 20–80% yield loss (Balachiranjeevi et al., 2019). Heavy infestation of BPH results in a dried-out rice stand phenomenon called 'hopperburn'. Locally known as 'bena perang'. BPH is also recognized as a vector of rice grassy stunt virus (RGSV) and rice ragged stunt virus (RRSV) (Jena & Kim, 2010). In late 2017, the Muda Agricultural Development Authority (MADA) granary experienced tremendous BPH infestation, involving a vast 800 ha (Khazanah Research Institute, 2019), followed by 213.70 ha in 2021 with 76.40 ha of hopperburn cases reported (Hashim, 2021). Similarly, in the Integrated Agricultural Development Area (IADA) Rompin rice granary, Pahang, BPH infestation had caused an extreme decline in rice production to as low as 2–3 t/ha (Khazanah Research Institute, 2019). Meanwhile, in Bagan Serai, Perak, about 79 ha of rice fields were damaged by BPH in January 2021, causing an RM24,000 loss to rice growers involved in the incident (Pauzi, 2021). In the following year, a group of 1,000 farmers in Yan, Kedah, incurred more than 50% yield losses after their crops were attacked by BPH (Awang, 2022). Meanwhile, in Pokok Sena, a total of 17 ha of rice fields were also afflicted by BPH, resulting in losses of nearly RM60,000 (Mansor, 2022).

Currently, farmers opt for synthetic insecticides to control BPH. Inevitably, intensive and excessive use of synthetic insecticides has caused several deleterious effects on human health, environmental degradation, and insect resurgence and resistance (Matsumura & Sanada-Morimura, 2010). Among the synthetic insecticides that have yielded resistant BPH populations are imidacloprid (Garrood et al., 2016), neonicotinoids carbamates, organophosphates, cyclodiene organochlorines, phenylpyrazoles (fiproles), pyrethroids-pyrethrins, and buprofezin (Khoa et al., 2018). Alternatively, a more sustainable BPH control method that renders less harmful environmental effects should be emphasized. Botanical pesticides, which are more environmentally benign, are currently being considered.

Various plant species, particularly medicinal plants, have numerously been

reported to possess insecticidal properties such as nicotine, rotenone, and pyrethrum (Said-Al Ahl et al., 2017; Stevenson et al., 2014). Nonetheless, weeds that are regarded as unwanted plants have always been understudied. Weeds are rather recognized as a nuisance, strong competitors, highly persistent, well adaptable to changing climate, and, to a certain extent, capable of exerting depressive effect upon the adjacent vegetations via allelopathic interactions. Equally, these special properties allow weeds to deter some insects and plant diseases, making them among the successful survivors in natural or disturbed ecosystems. Therefore, weeds could offer great potential as plant-based biopesticides as they are relatively biodegradable and can become a crucial alternative to harmful synthetic chemicals.

In Malaysia, *Ageratum conyzoides* (L.), *Chromolaena odorata* (L.) R. M. King & H. E. Robins, and *Mallotus paniculatus* (Lam.) Mull. Arg. are common weeds in agricultural ecosystems. *Ageratum conyzoides* plant extracts effectively controlled cowpea weevil (Gbolade & Adebayo, 1993) and grasshopper (Ingrid et al., 2020). Whereas *C. odorata* successfully caused mortality in rice weevils (Acero, 2014) and blackflies (Matur & Davou, 2007). In addition, *M. paniculatus* and other Euphorbiaceae were also reported to have insecticidal properties against *Plutella xylostella* L. (Uma & Kumar, 2009). The cause of insect mortality from these weed species was due to bioactive compounds such as saponin, alkaloids, flavonoids, terpenoids, and tannins (Udebuani et al., 2015). Nonetheless, information regarding the insecticidal activities of these three weed species against BPH is still lacking.

Plant bioactive compounds could be extracted by conventional or non-conventional methods (Azwanida, 2015). A very common method used to extract plant secondary metabolites is solid-liquid solvent extraction, also known as Normal Soaking Extraction (NSE) or maceration with the use of organic solvent, i.e., methanol and hexane (Tiwari et al., 2011; Zhang et al., 2018). These similar polarities extraction concepts contain a complex mixture of plant metabolites, which can later be identified by chemical analysis. Gas chromatography-mass spectrometry (GC-MS) is one of the instruments used to quantify the presence of the bioactive compounds in the plant extract. GC separates many volatile and semi-volatile compounds but does not selectively detect them, while MS is capable of selectively detecting many compounds but not always separating them (Sneddon et al., 2007).

Insect mortality bioassay is advantageous when specific compounds from the plant extract are recognized for their detrimental potential. Additionally, this practice offers valuable insights, especially when conducting insecticide formulation. The effectiveness of plant extract against pests could be improved by developing a formulation of bio-insecticide prior to commercialization into the market. A pesticide formulation is a chemical mixture comprising inert materials and active

ingredients (a.i.) that effectively controls the target pests (Hazra & Purkait, 2019). Solvents, carriers, and adjuvants/surfactants are the common inert ingredients that are intentionally added into the formulation to aid the pesticide stability, handling, safety, and ease of application and increase pesticide retention-absorption capacity (Hazra & Purkait, 2019). An active ingredient is a substance that prevents, kills, or repels a pest. The introduction of inert ingredients may aid in applying the active ingredient (National Pesticide Information Center, n.d.). Emulsion formulation is an example of a pesticide formulation. It can be macro, micro, or nano-emulsion, oil-in-water, or water-in-oil. The characteristics of the emulsion are based on the ratio of the components during the emulsion preparation and the compatibility of the emulsion with the active ingredients.

Thus, the present study explores the insecticidal potential of the selected weed species in solvents having different polarities towards BPH. The bioactive compounds in the *C. odorata* methanolic extract were also characterized, and comparable bio-insecticide formulations were developed and evaluated for their bio-efficacy against BPH.

## MATERIALS AND METHODS

### Insect Collection and Rearing

Heong et al. (2013) adopted insect collection and rearing methods. Approximately 50 healthy and unparasitized female adults of short-winged (brachypterous) BPH (or alternatively about 100 nymphs when the number of adults was insufficient) were collected randomly using custom-made manual mouth aspirator in rice fields in Pendang, Kedah. The stock culture of BPH was maintained in a glasshouse, Ladang 10, Universiti Putra Malaysia, and had no exposure to any pesticide. The culture was maintained on MR219 rice seedlings in an individual mylar rearing cage (45 × 90 × 45 $cm^3$), having a day/night temperature of 33/20 ± 2°C and an average of 13 hr daylight.

### Collection of Plant and Extract Preparation

Leaves of *A. conyzoides*, *C. odorata*, and *M. paniculatus* were collected from the mature plants within the Universiti Putra Malaysia. The leaves were separated and washed thoroughly under running tap water and oven-dried at 40°C (± 2°C) for 72 hr. Dried leaves were pulverized by an electric grinder (LB10S, Waring, USA). The extraction process was done by soaking the powdered sample in two solvents, which are methanol and hexane, at 1:10 w/v of solid-liquid ratio for 72 hr with constant agitation by an orbital shaker at 100 rpm. After that, the extract solutions were filtered (Whatman Nylon Membrane Filters 0.45 µm, USA), and the filtrates were concentrated by using a rotary vacuum evaporator (R-215; BUCHI, United Kingdom) at 100 rpm and 40°C. Concentrated extracts were transferred into 30 ml glass vials wrapped with aluminum foil and kept in a -20°C freezer for further use. Extraction processes were done in triplicates.

**Insecticidal Activity of Selected Weeds Extracts**

Preliminary contact toxicity tests were executed against the 3rd and 4th instar BPH nymphs to determine a range of concentrations of the plant extracts that cause 5–99% mortality (Nuryanti et al., 2018). A 2% crude extract concentration stock solution was prepared by adding 50 ml of methanol to 1 g of crude extract. From the stock, a series of concentrations (500, 1,000, 1,500, and 2,000 ppm) were prepared for all three weed species by adding distilled water according to the equation given below:

$$C1V1 = C2V2$$

where, C1 = Concentration of stock solution (ppm), C2 = Desired concentration to prepared (ppm), V1 = Volume from stock solution (ml), and V2 = Desired volume to prepared (ml), respectively.

From the preliminary mortality bioassay result, a new series of concentrations for *A. conyzoides* and *C. odorata* methanol extracts (100, 500, 1,000, 5,000, and 10,000 ppm, respectively) and *M. paniculatus* methanol extract (5,000, 6,000, 7,000, 8,000, 9,000, 10,000, and 12,000 ppm, respectively) were prepared as detailed above to determine the toxicity effect of the crude extracts on BPH nymphs.

A mortality bioassay was carried out using the plant dip method described in Test Method No. 005 (Nauen, n.d.). Bacteriological agar No. 1 was prepared and cooled until semi-solidified (approximately 37°C). The semi-solid agar was poured about 100 ml into each pot containing 15–21 days-old rice seedlings to cover the soil surface. After the agar solidification, the pots were reverted and dipped completely into the treatments for 10 s. Then, all the rice seedlings were allowed to dry for 60–90 min, depending on the ambient relative humidity and placed into an individual cylinder cage (22.50 cm height, 8.50 cm diameter) to prevent insects from escaping.

The 3rd and 4th instar BPH nymphs were collected from the rearing cage (nymphs cage) using a mouth aspirator. Ten nymphs were collected and left to starve for one hour. Later, all the starved nymphs were released onto the treated rice seedlings, and the cylinder cage was closed with a lid to prevent the nymph from escaping. Observation of nymphs' mortality was done at 24, 48, and 72 hr. Nymphs were considered dead if they did not respond to gentle probing with a fine sable brush. The control mortalities were corrected using Abbott's (1925) formula as below:

$$\text{Corrected mortality (\%)} = [\text{\% Test mortality} - \text{\% Control mortality} / 100 - \text{\% Control mortality}] \times 100\%$$

**Preparation of Emulsion Formulation**

A non-ionic alkanolamide surfactant, Emersense® AM 8025 (Emery Oleochemicals (M) Sdn. Bhd., Malaysia), was incorporated in the surfactant phase of the formulation to increase the solubility of the oily component in the emulsion based on miscibility studies of the formulation. Three types of oil-based carriers, namely Edenol®

SP100 and Edenor® (Emery Oleochemicals (M) Sdn. Bhd., Malaysia) and palm kernel oil ester (PKOE) (Department of Chemistry, Faculty of Science, Universiti Putra Malaysia) were used to develop the bio-insecticide formulations. The emulsions were prepared using purified water (Elga Labwater, 18 m) by titration.

Surfactant and carrier were mixed in a 15 ml centrifuge tube at ratios of 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 9:1, and 0:10 (w/w). A non-ionic alkanolamide surfactant Emersense® AM 8025 (Emery Oleochemicals (M) Sdn. Bhd., Malaysia) was used as the surfactant, while PKOE (Department of Chemistry, Faculty of Science, Universiti Putra Malaysia), Edenol® SP100, and Edenor® (both from Emery Oleochemicals (M) Sdn. Bhd., Malaysia) were used as the oil-based carriers to develop the bio-insecticide formulations. Then, deionized water (5% [w/w]) was added by titrating into the mixtures of surfactant and carrier until 95% water content was achieved in the emulsion system.

Analytical balance (Mettler Toledo Model Dragon 204, Spain) weighed each component. The prepared compositions were homogenized using a vortex mixer (Model VTX-3000 L, Japan) and allowed to mix for approximately 3–5 min until equilibrium was achieved. The emulsions were homogenized, followed by centrifugation at 120 × *g* and 25°C for 30 min (Flanangan et al., 2006). The results obtained from the experiment were subjected to Chemix version 3.5 phase diagram plotter (United Kingdom), a software used to construct a pseudoternary phase diagram. In the pseudoternary phase diagram, several points were chosen within the isotropic region (one-phased) with the criteria of surfactant being <30% and incorporation of 5% *C. odorata* methanol leaf extract into the emulsion as the active ingredient (Table 1). The formulation that was miscible with *C. odorata* leaf extract (5%) and retained a one-phase appearance proceeded for characterization and insect bioassay.

## Characterization of Emulsion Formulation

The stability test was carried out with a formulation that was miscible with *C. odorata* extract and retained the one-phase appearance. EM-PKOE emulsion incorporated with *C.*

Table 1
*Ingredients used in the ternary phase diagram*

| Compound | Trade name | Class |
|---|---|---|
| Palm kernelamide DEA | Emersense® AM 8025 | Surfactant |
| N. A. | Edenol® SP100 | Carrier |
| Palm oil methylester | Edenor® | Carrier |
| Palm oil alkylester | Palm kernel oil ester | Carrier |
| Water | N. A. | Water |
| *Chromolaena odorata* methanol leaf extract | N. A. | Active ingredient |

*Note*. N. A. = Not available

*odorata* extract was centrifuged at 120 × *g* for 30 min and kept at room temperature (25 ± 2°C) for 30 days and at 54°C for 14 days in accordance with the Food and Agricultural Organization (FAO) as a standard evaluation for agrochemical products to show the stability of the formulations in the tropical climate (Yusoff et al., 2021). The physical appearance of the emulsion was visually investigated.

Particle size, polydispersity index (PDI), and zeta potential analysis were performed on freshly prepared EM-PKOE emulsion. The formulation was diluted with deionized water in a falcon tube and gently mixed with the analyses. One (1) ml from the sample was pipetted into a quartz cell with a surface area of 1 $cm^2$ and placed in a Zetasizer Nano-ZS (Malvern Instruments Ltd., United Kingdom). The particle size of the formulation was determined using dynamic light scattering (DLS) to capture the Brownian motion of the dispersed phase, and the zeta potential (surface charge) of the emulsion was determined via laser doppler electrophoresis (LDE). The PDI was calculated from the photo correlation spectroscopic analysis via electrophoretic light scattering (ELS).

The viscosity test of the one-day-old formulation was measured at room temperature (25 ± 1°C) using a digital viscometer (NDJ-5S, ATO, China). Approximately 30 ml of the stock formulation was filled in the viscometer cup, followed by a rotation speed of 12 rpm at 20,000 to 100,000 millipascal-seconds (mPa.s). The measurements were taken in triplicate.

### *Bioassay Test for Emulsion Formulation*

Mortality bioassays for emulsion formulation were conducted using the method described above with minor modifications. Five treatment concentrations (100, 500, 1,000, 5,000, and 10,000 ppm) were diluted from the EM-PKOE emulsion. Each pot containing MR219 rice plants was fully sprayed for approximately 10 s and labeled accordingly. In about an hour, bacteriological agar was filled into the pot when the treated plants were completely dried. Ten 3$^{rd}$ and 4$^{th}$ instar nymphs were released into each treated pot and encased in a plastic cylinder. Four replicates of the positive controls (Neemix 4.5® [SURECROP SDN. BHD., Malaysia] and Regent® 50SC [Bayer Co., Malaysia]) and the negative control (distilled water) were performed. The mortality of nymphs was assessed at 6, 12, 18, 24, 30, 36, 42, and 48 hr after treatment.

### GC-MS

The chemical constituents of *C. odorata* methanol leaf extract were determined using the GC-MS technique (Shimadzu GC-2010 Plus, Japan), equipped with a Rxi-5ms capillary column (30.00 m × 0.25 mm inner diameter × 0.25 µm film thickness) coupled with Shimadzu GCMS-QP2010 Ultra (Japan). The oven temperature was programmed from 50 to 300°C at a rate of 3°C/min and held for 10 min. The injector temperatures were held at 250°C. The sample injected into the injector was split mode with a ratio 10. The pressure was applied at a constant flow rate of 0.80 ml/min. An electron ionization system with an

ionization voltage of 0.91 kV was used for GC-MS detection. The chemical compounds of *C. odorata* methanol leaf extract were identified by comparing their retention time and mass spectra with those recorded in databases of the National Institute of Standards and Technology (NIST) on the GC-MS system and expressed as a percentage by peak area.

### Statistical Analysis

All data were subjected to analysis of variance (ANOVA, SAS statistical software version 9.4), followed by means separation by least significant difference (LSD) test with square root transformation for extraction yields, while means separation by Tukey's multiple range test with arcsine transformation for insect mortality bioassay. The average of nymphs' mortality data was subjected to probit analysis (EPA Probit Analysis Software Program version 1.5) to calculate the $LC_{50}$. The values were expressed as means ± standard error of four replicates. Results with $p<0.05$ were statistically significant. The graphs were best fitted into a 4-parametric sigmoidal hill curve (SigmaPlot 14) according to the $R^2$ value.

## RESULTS AND DISCUSSION

### Extraction Yield of Plant Crude Extracts

It was observed that the extraction yields in all plant species were comparable in both solvents. Nonetheless, methanol always results in higher extraction yields in all species than hexane, as shown in Table 2.

Evidently, higher leaf extract in all selected weed species was recorded in methanol over hexane, as Prajapati et al. (2014) reported on the higher plant extract in methanol than in hexane. Solvent type, extraction method, and sample preparations are among the main factors that eventually influence the outcome of the final extracts (Azwanida, 2015). As an intermediate polar solvent, methanol also tends to extract polar compounds and some non-polar compounds (Tambellini et al., 2013). Similarly, it was also observed that high polar solvents such as methanol were more effective in extracting bioactive compounds in *Garnicia atriviridis* (Al-Mansoub et al., 2014) and *Hibiscus micranthus* (Begashaw et al., 2017) over the non-polar solvents.

### Mortality Bioassay of Plant Crude Extract

In the preliminary study, the 3rd and 4th instar-nymphs of BPH were used instead

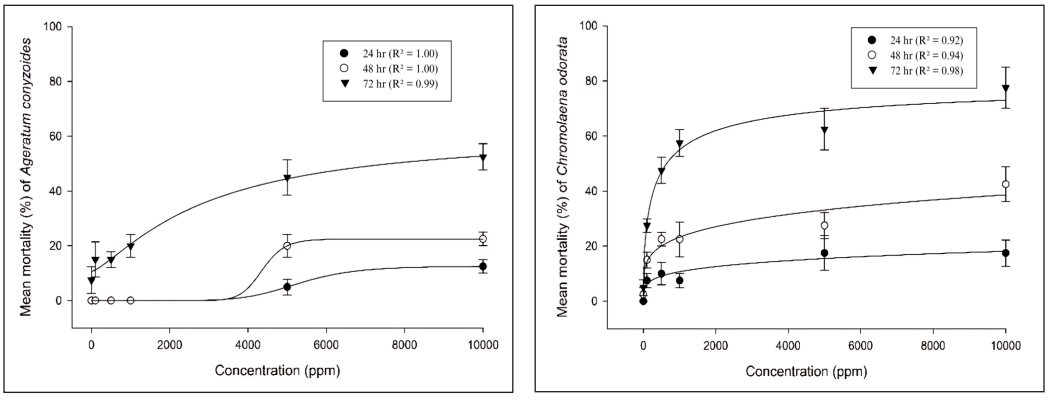
Table 2
*Mean separation of extraction yield for different weed species in different solvent*

| Factor | *P* value |
|---|---|
| Plant | ns |
| Solvent | <0.0001*** |
| Plant × Solvent | ns |
| Factor | Mean of extraction yield (%) |
| Plant | |
| *Ageratum conyzoides* | 11.19 ± 3.61a |
| *Chromolaena odorata* | 10.76 ± 3.42a |
| *Mallotus paniculatus* | 8.77 ± 2.69a |

*Note*. *** = Highly significant, ns = Non-significant. Means followed by the same letters (among plants and solvents) are not significantly different; Least significant difference test at $p = 0.05$

of the adult because of the rapid damage caused by the nymphs compared to the adult. Saxena and Khan (1985) highlighted that when studying neem extract using different solvents (aqueous, ethanol, and hexane), conducting bioassays with early instar nymphs can provide more practical insights, especially in field experiments. Similarly, Senthil-Nathan et al. (2007) recommended using 3rd and 4th instar nymphs to target early developmental stages when conducting bioassays for BPH control, which is in line with the current study. The methanol extract of *A. conyzoides* tested against the BPH showed a highly significant mortality interaction between treatment concentration and exposure time (Figure 1). As treatment concentrations and exposure times increased, nymphs' mortality also rose. A remarkable significant in mortality was observed between the two highest concentrations (5,000 and 10,000 ppm) and the three lowest concentrations (100, 500, and 1,000 ppm). Mortality rates rose progressively from 7.50% at the control to 15% at both 100 and 500 ppm, and then to 20, 45, and 52.20% at increasing concentrations, reaching the highest level at 10,000 ppm after 72 hr of exposure. Regardless of concentration, more time was needed by the *A. conyzoides* methanol leaf extract to exhibit 50% mortality to BPH. Nonetheless, low mortality (<60%) was observed, even at the highest concentration (10,000 ppm), stipulating that *A. conyzoides* only possesses a little toxicity towards the BPH. It was also noticed that at lower concentrations (1,000 ppm and below), the mortality was slow to develop, whereas the extract only showed its effect 72 hr after exposure time.

Similarly, a highly significant interaction between the treatment concentration and exposure time was observed in *C. odorata*. Nymph mortality increased substantially from 24 to 72 hr after exposure as treatment concentration increased (Figure 1). Less than 50% mortality occurred at 24 and 48 hr of




(a) (b)

*Figure 1*. Interaction of concentration-time-mortality of the 3rd - 4th instar brown planthopper nymphs treated with (a) *Ageratum conyzoides* and (b) *Chromolaena odorata* methanol extract. The curves were fitted into a 4-parameteric sigmoidal hill curve using SigmaPlot 14 software. The standard error of the means was presented by vertical bars (n = 4)

exposure, even at the highest concentration (10,000 ppm). Mortality increased gradually at 72 hr of exposure time, where the highest nymph mortality (77.50%) was achieved at the highest concentration (10,000 ppm), indicating that *C. odorata* possesses a promising insecticidal compound toward the BPH.

*Mallotus paniculatus* methanol leaf extract was screened against BPH in eight concentrations, including the control. Highly significant mortality was observed between the treatment concentrations and between the exposure times (Table 3), while no significant interaction effect was observed. Low mortality was observed in the lowest four concentrations, from 0 (control) to 7,000 ppm, with less than 45% mortality. At the same time, the insecticidal effect was observed to be high (>45% mortality) in the four highest concentrations (8,000 to 12,000 ppm). Extension of the exposure time also increased the killing effect of the plant extract to approximately 1.8-fold from 24 to 72 hr.

The insecticidal activity of different plant extracts varies significantly, depending on the plant species and compatibility of the extraction solvent with the plant. Furthermore, insecticidal activity increased when treatment concentration increased simultaneously with the exposure times (Ahmed et al., 2020). Most synthetic insecticides can cause acute mortality. On the other hand, insects treated with plant extract had a slower mortality rate, whereas the extract did not cause death acutely. According to Ali et al. (2017), this phenomenon was due to some behavioral or physiological alterations, commonly known as sublethal or non-lethal effects experienced by the test insect. The dose/concentration the insect was exposed to would differ greatly over space and time. In a study conducted by Senthil-Nathan

Table 3
*Mean mortality of 3rd and 4th instar brown planthopper (BPH) nymphs treated with* Mallotus paniculatus *methanol extract*

| | Percentage (%) mortality of BPH | | | |
|---|---|---|---|---|
| **Treatment (ppm)** | **24 hr** | **48 hr** | **72 hr** | ***Mean regardless of time ± SE** |
| Control (Water) | 2.50a | 7.50a | 7.50a | 5.83 ± 1.93e |
| 5,000 | 20.00b | 30.00ab | 47.50ab | 32.50 ± 4.79d |
| 6,000 | 25.00a | 35.00a | 50.00a | 36.67 ± 5.27cd |
| 7,000 | 32.50b | 50.00a | 50.00a | 44.17 ± 3.13bcd |
| 8,000 | 32.50c | 47.50b | 62.50a | 47.50 ± 5.11abc |
| 9,000 | 35.00b | 47.50ab | 65.00a | 49.17 ± 4.84abc |
| 10,000 | 45.00b | 57.50ab | 70.00a | 57.50 ± 4.29ab |
| 12,000 | 47.50a | 60.00a | 72.50a | 60.00 ± 5.08a |
| *Mean regardless of treatment ± SE | 30.00 ± 2.94c | 41.88 ± 3.34b | 53.13 ± 4.05a | |

*Note*. Means followed by the same alphabet within columns (hour) are not significantly different by Tukey's test at $p = 0.05$. Means followed by the same alphabet, regardless of time (within column) or treatment (within row), are not significantly different by Tukey's test at $p = 0.05$

et al. (2009), the effective concentration of two botanical insecticides, Parker Oil™ (Parker Hannifin Corporation, USA) and Neema® (Neema International, USA) took more than 48 hr to kill 80% of the BPH. In the current study, the inhibitory effect of the plant extracts on BPH was observed every 24 hr for 3 consecutive days. After 72 hr, the rice plants in the control treatment (distilled water) also began to die due to rapid consumption by the BPH, resulting in a decrease in the BPH due to the depletion of food sources. It is also equally notable that all the formulated commercial insecticides have a specific active ingredient/premix of active ingredients, which have been proven through intensive evaluation to possess effective toxicity towards the target pest insect at the registered recommended rate. Hence, an investigation of the chemical compounds in the selected weed species was also done as an antecedent step in isolating the potential primary active ingredient candidate for bio-insecticide.

## $LC_{50}$ of 3rd and 4th Instar Nymphs of BPH in Methanol Extracts

$LC_{50}$ is a concentration of a substance in a controlled environment that is expected to kill 50% of tested organisms in a given population (Duffus, 1993). An $LC_{50}$ assessment was done on the mortality at 72 hr after exposure to the plant extracts to evaluate the effective toxicity effect of the methanol extracts. The results demonstrated that *C. odorata* significantly recorded the lowest $LC_{50}$ value as compared to *M. paniculatus* and *A. conyzoides* (Table 4).

In a study by Lawal et al. (2014), the methanol extract of *C. odorata* becomes highly toxic even at very low concentrations, resulting in an $LC_{50}$ value of 0.0039% (39 ppm) to kill *Sitophilus zeamais*. In comparison with another study by Matur and Davou (2007), *C. odorata* plant extract was able to cause low $LC_{50}$ value against *Simulium* larvae, which was 0.001 mg/ml (100 ppm) concentration. On the contrary, methanol extract of *Ocimum gratissimum* only caused 73.92% mortality at 10,000 ppm (M. S. Kumar et al., 2017), similar to what was observed in this study for *C. odorata*. In plant extraction by solvent, the ability of certain plants to exhibit high mortality at a lower rate was due to several factors, such as pre-extraction preparation (plant part and sample preparation) and solvent choices (Azwanida, 2015). The solvent selection was of great significance (Khan et al., 2017), as it would ultimately dictate the extraction compounds that play a key role in affecting insect bioassays. This selection

Table 4
*Median lethal concentration ($LC_{50}$) value after 72 hr exposure*

| Sample | Slope ± SE | $LC_{50}$ (ppm) | 95% confidence limits (ppm) | Chi-square ($X^2$) |
|---|---|---|---|---|
| *Ageratum conyzoides* | 0.86 ± 0.28 | 21,940a | 9,469 ~ 325,720 | 0.99 |
| *Chromolaena odorata* | 0.63 ± 0.14 | 977b | 368 ~ 2,151 | 1.46 |
| *Mallotus paniculatus* | 2.12 ± 0.69 | 6,549a | 4,074 ~ 8,039 | 0.90 |

*Note*. $LC_{50}$ value with the same alphabet are not significantly different by Tukey's test at $p = 0.05$

not only determines the type and quality of extracted bioactive compounds but also directly influences insect mortality during the bioassay process (Fotsing et al., 2021).

## Emulsion Formulation

The pseudoternary phase diagram was constructed to compare the surfactant, oil, and water ratio to obtain a single-phase (isotropic) region while conducting the emulsion formulation. Three pseudoternary phase diagrams were attained, comprising three different oils as carriers with similar surfactant types (Table 5). The three-component system is shown in Figure 2 (Emersense® AM 8025/PKOE/water), Figure 3 (Emersense® AM 8025/Edenor®/water) and Figure 4 (Emersense® AM 8025/Edenol® SP100/water). A continuous single-phase diagram of the isotopic region was observed in the green area, which is <20% of the total area for the three different formulations, while the blue/pink/orange region refers to milky and multi-layer (multi-phase). Emersense® AM 8025/PKOE/water system and Emersense® AM 8025/Edenor®/water system exhibited similar percentages of isotropic region (Figures 2 and 3). Nevertheless, Emersense® AM 8025/Edenol® SP100/water system resulted in a very trifling isotropic region (Figure 4). The solubilization capacity and areas of the emulsion systems were due to the structural similarity between the lipophilic

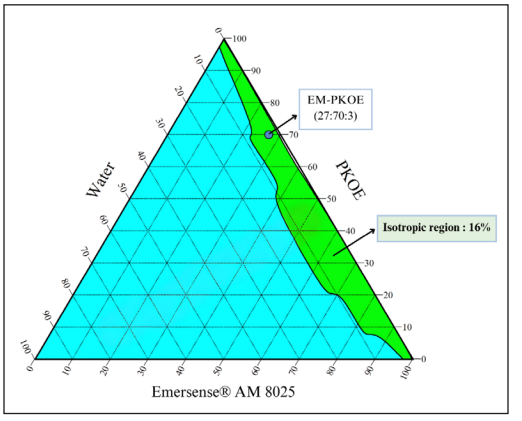



*Figure 2*. Phase diagram of Emersense® AM 8025: PKOE: Water system
*Note*. EM-PKOE = Emersense® AM 8025/palm kernel oil ester/water

Table 5
*Percentage (w/w) compositions of surfactants, oil, and water contained in the emulsion formulations system without plant extract*

| Formulation (w/w) | Emersense® AM 8025[a] (%) | PKOE[b] (%) | Edenor®[c] (%) | Edenol® SP100[d] (%) | Water[e] (%) |
|---|---|---|---|---|---|
| EM-PKOE | 27 | 70 | - | - | 3 |
| EM-ED$_1$ | 30 | - | 20 | - | 50 |
| EM-ED$_2$ | 25 | - | 15 | - | 60 |
| EM-ED$_3$ | 20 | - | 10 | - | 70 |
| EM-ED$_4$ | 15 | - | 5 | - | 75 |
| EM-EDSP$_1$ | 29 | - | - | 11 | 60 |
| EM-EDSP$_2$ | 25 | - | - | 5 | 70 |

*Note*. [a] = Non-ionic surfactant; [b,c,d] = Oil carriers; [e] = Solvent; EM-PKOE = Emersense® AM 8025/palm kernel oil ester/water; EM-ED = Emersense® AM 8025/Edenor®/water; EM-EDSP = Emersense® AM 8025/Edenol® SP100/water

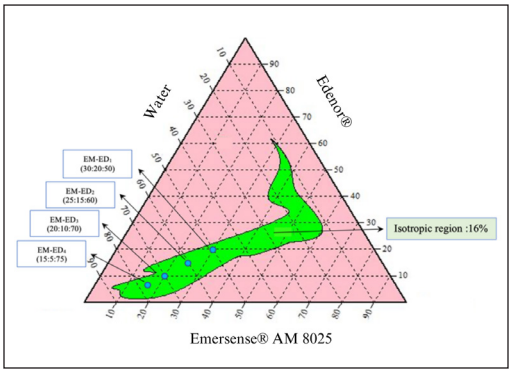



*Figure 3*. Phase diagram of Emersense® AM 8025: Edenor®: Water system
*Note*. EM-ED = Emersense® AM 8025/Edenor®/ water

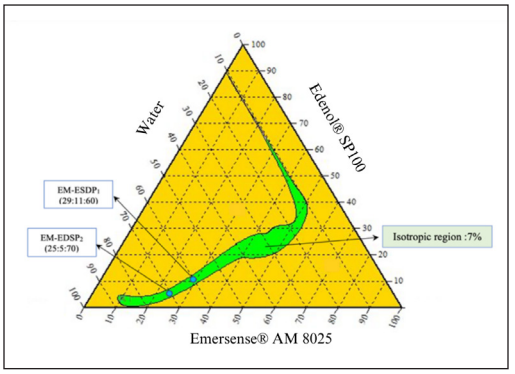



*Figure 4*. Phase diagram of Emersense® AM 8025: Edenol® SP100: Water system
*Note*. EM-EDSP = Emersense® AM 8025/Edenol® SP100/water

tail of surfactant and the oleyl group of the PKOEs (Mahdi et al., 2011).

There are three types of surfactants: ionic, anionic, and non-ionic. The most commonly used surfactant in pesticide formulation is non-ionic (Appah et al., 2020). A non-ionic surfactant was chosen in this study because of the less toxicity and irritation it imposes upon plants, animals, and humans, as well as the impact on the environment (Azeem et al., 2009; Pulce & Descotes, 1996). The required amount of surfactant also plays an important role in conducting emulsion formulation as a high concentration of surfactant could lead to phytotoxicity and also increase the production cost because surfactants are the most expensive element in an emulsion formulation compared to oil (Pratap & Bhowmick, 2008). Surfactants below 30% were suitable for emulsion formulation, especially in formulating microemulsions (Pratap & Bhowmick, 2008) and nanoemulsions (Choupanian et al., 2017).

Several points were selected from the isotropic region (green-colored region) according to the criteria of an adequate amount of surfactant (<30%). One point from Emersense® AM 8025/palm kernel oil ester/ water coded as EM-PKOE, four points from Emersense® AM 8025/Edenor®/water coded as EM-ED, and two points from Emersense® AM 8025/Edenol® SP100/water coded as EM-EDSP were selected within the isotropic region. Based on the chosen points, only EM-PKOE emulsion had the least water content (3%) and the highest oil content (70%) in the formulation. In contrast, emulsions containing either Edenor® or Edenol® SP100 were observed to have higher water content (>50%) than oil content (<20%).

According to this finding, the methanol crude extract of *C. odorata* showed low miscibility when mixed with water alone. Thus, the incorporation of 5% *C. odorata* methanol crude extract as the active ingredient into the seven emulsion systems was investigated. Among the seven points from the three different emulsion systems,

only EM-PKOE emulsion appeared compatible with the 5% *C. odorata* methanol crude extract as it achieved a stable emulsion with no phase separation. In contrast, EM-ED and EM-EDSP showed incompatibility with 5% *C. odorata* methanol crude extract as they failed to remain single-phased. The compatibility of the extract when incorporated with the emulsion might be due to the compounds present in the extract. It is well known that oils are widely used as emulsion-type pesticide adjuvants to enhance the spread of droplets on plant surfaces with the possibility to penetrate lipophilic regions in leaf cuticles, thus increasing absorption of leaf extract into plant cells (Wang et al., 2018).

## Characterization of the Macroemulsion Formulation

### *Stability of Formulation*

EM-PKOE emulsion, which contained 5% *C. odorata* extract, was tested for its stability at room temperature (25 ± 2°C) for 30 days and thermostability at 54°C for 14 days. Based on visual observation, there was no phase separation, creaming, or sedimentation in the emulsion at room temperature and after the heat test (54°C). Hence, this emulsion exhibited good stability, which met the requirements for insecticide formulation according to the Food and Agriculture Organization of the United Nations and the World Health Organization (2010).

### *Zeta Potential*

The zeta potential determined for EM-PKOE using LDE were found to be negatively charged (-62.70 mV). Zeta potential is often used as an indicator of droplet stability, where values more positive than +30 mV and more negative than -30 mV (Han et al., 2008) exhibit strong repelling force, indicating good stability against coalescence (Kadu et al., 2011). A net charge of either sign on the particle surface repels the particles electrostatically, preventing aggregation, coagulation, or flocculation to a certain extent (Sis & Birinci, 2009). Therefore, the zeta potential determined in this study demonstrates emulsion stability, as it exceeds -30 mV. The zeta potential is the difference in potential between the mobile dispersion medium and the stationary layer or fluid in which the dispersed particle is suspended (Lu & Gao, 2010). In other words, it is the assessment or determination of a colloidal system's surface charge (Shnoudeh et al., 2019).

### *Particle Size and PDI*

The particle size distribution of diameters of the dispersed phase of EM-PKOE was determined using the Malvern instrument and the DLS technique. Based on the analysis, the mean particle size of the EM-PKOE emulsion was 444.40 nm in diameter, considered a conventional emulsion or macroemulsion. Macroemulsions have particle sizes >200 nm in diameter, and they are optically turbid as in EM-PKOE because the droplet size is comparable to the wavelength of light, hence scattering the incident light and making it appear opaque. In addition, macroemulsion is kinetically stable and thermodynamically metastable. The advantage of this emulsion

type is also comparable to nanoemulsion, where it is very stable to temperature and pH changes (Aswathanarayan & Vittal, 2019).

The determination of particle size in an emulsion is one of the very important physical characteristics because it can distinguish the emulsion types and the stability of the emulsion (Mudalige et al., 2019). A large particle size in an emulsion was most likely due to a higher oil content, resulting in a larger droplet size in the emulsions (Zheng et al., 2020). The higher oil content, therefore, describes the emulsion type as a "water-in-oil" (w/o) emulsion where water is the dispersed phase that is distributed into the continuous phase (oil). Moreover, the droplet size distribution will also contribute to the determination of the PDI of an emulsion.

The PDI is basically a depiction of the size distribution heterogeneousness by the instruments of DLS within a sample. This index is dimensionless with value ranges from 0.00 to 1.00 for a sample that is totally uniform/homogeneous and for a sample that is not perfectly uniform/not homogeneous in particle size distributions accordingly (Danaei et al., 2018). In this study, the PDI analyzed for EM-PKOE was 0.60. According to Nobbmann (2017), a PDI value >0.40 is categorized as a broad polydisperse distribution, meaning the sample particles varied in size. Polydispersion may arise because of the distribution or agglomeration of a sample during isolation or analysis (Mudalige et al., 2019) and might be due to the solvent used during the dilution of the sample, as mentioned by Motwani et al. (2006).

The higher the PDI, the larger the particle size. The particle size distribution is reflected in the PDI value as well. Samples with a larger range of particle sizes have higher PDI values, whereas samples with equally sized particles have lower PDI values (Masarudin et al., 2015). There is no PDI limit because the purpose of the formulation determines it. For example, it is feasible to have a low PDI value since it reflects particle dispersity in a homogeneous sample, which means that the particles are smaller and more uniform, and therefore, if penetration is necessary to infiltrate a cell, it is more efficient (Danaei et al., 2018).

### *Viscosity*

The viscosity test that was carried out on EM-PKOE emulsion containing 5% *C. odorata* extract showed a range of 33.09 to 35.29 mPa.s, which is considered very viscous, while the viscosity of water is only about 0.90 mPa.s at room temperature (Foliadi et al., 2018). Viscosity depends on surfactant type, surfactant-to-oil (S/O) ratios, and oil concentration. Increased S/O and oil concentration will result in high viscosity (Chanana & Sheth, 1995). Similarly, reducing the amounts of surfactant or decreasing water volume can also increase interfacial tension between water and oil, producing a more viscous emulsion (Marzuki et al., 2019).

## Mortality Bioassay of Emulsion Formulation Containing Crude Extract

Toxicity effect determination of EM-PKOE macroemulsion containing 5% *C. odorata*

leaves extract was performed against the 3rd and 4th instar nymphs of BPH. Based on the analysis, BPH cumulative mortality was significantly influenced by the treatment concentration and the days of exposure. Further analysis was carried out to observe the interaction between the two factors (treatment concentrations and days of exposure), and a highly significant interaction was recorded between the treatment concentrations and days of exposure. Nymph mortality increased as treatment concentrations and days of exposure were increased. Higher concentration and more time were needed to achieve higher nymph mortality.

Based on these findings, the formulated EM-PKOE macroemulsion with *C. odorata* leaf extract had a toxic effect against BPH 3rd and 4th instar nymphs. Nymph mortality escalated as exposure time increased regardless of the treatment concentrations (Table 6). Furthermore, nymphs' mortality was also influenced by the macroemulsion concentration, where higher concentration resulted in a higher mortality percentage regardless of the exposure time. Hence, it can be concluded that treatment concentration and exposure time apparently showed significant interaction, where an increase in exposure time and treatment concentration would cause an increase in mortality percentage (Bouda et al., 2001).

The EM-PKOE emulsion with *C. odorata* methanol extract displayed higher efficacy against the BPH nymphs, especially at higher concentrations (5,000 and 10,000 ppm) as compared to the positive controls, namely Regent® (fipronil) and Neemix® (azadirachtin), the commercial chemical and botanical insecticide, respectively. Overall, during the final observation (48 hr), there was no significant mortality among the four higher concentrations (500, 1,000, 5,000, and 10,000 ppm) and the positive controls, where the EM-PKOE emulsion was able to kill >80% of the nymphs at lower concentration of 500 ppm. Interestingly, at 1,000 ppm, mortality of the emulsion was comparable to that of the positive controls, which was also evident in every hour of observation. Fipronil used in this study is a broad-spectrum insecticide, which is highly effective against sucking and chewing insects. It has been widely used for the control of many species of soil and foliar insects of various agricultural crops, but indiscriminate use of this insecticide in recent times has resulted in the development of resistance in planthoppers (N. Kumar et al., 2019). On the other hand, azadirachtin is a potent antifeedant (feeding deterrent) botanical insecticide that often results in starvation, ultimately killing the insect (Heong et al., 2013).

The emulsion formulation greatly affected and influenced the nymphs' mortality in this study. The incorporation of *C. odorata* into the EM-PKOE emulsion substantially improved the extract's efficacy. As an example, at the highest concentration of 10,000 ppm, a total control (100%) was improved by 22.50% at a shorter exposure time (48 hr) in the formulated emulsion as compared to the *C. odorata* crude extract alone (77.50% mortality after 72 hr of exposure) against the BPH nymphs.

Table 6
*Interaction of treatment concentration-time-mortality of 3rd and 4th instar nymphs of Nilaparvata lugens treated with EM-PKOE macroemulsion containing 5% Chromolaena odorata extract*

| | Percentage (%) mortality of BPH | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | **6 hr** | **12 hr** | **18 hr** | **24 hr** | **30 hr** | **36 hr** | **42 hr** | **48 hr** | ***Mean regardless of time ± SE** |
| Control (Fipronil) | 52.50b | 60.00bc | 75.00b | 82.50ab | 90.00a | 95.00a | 97.50a | 97.50a | 81.30 ± 3.20bc |
| Control (Azadirachtin) | 45.00b | 70.00b | 77.50b | 95.00a | 95.00a | 97.50a | 97.50a | 97.50a | 84.40 ± 3.42b |
| Control (Water) | 0.00c | 0.00d | 0.00e | 0.00c | 0.00d | 5.00d | 10.00c | 12.50c | 3.40 ± 1.15f |
| 100 ppm | 7.50c | 15.00d | 15.00d | 17.50c | 22.50c | 25.00c | 25.00c | 30.00b | 19.70 ± 2.03e |
| 500 ppm | 37.50b | 47.50c | 55.00c | 60.00b | 62.50b | 72.50b | 77.50b | 85.00a | 62.20 ± 3.32d |
| 1,000 ppm | 55.00b | 65.00bc | 72.50b | 85.00ab | 85.00a | 85.00ab | 92.50ab | 92.50a | 79.00 ± 2.43c |
| 5,000 ppm | 77.50a | 90.00a | 97.5.0a | 100.00a | 100.00a | 100.00a | 100.00a | 100.00a | 95.60 ± 1.62a |
| 10,000 ppm | 85.00a | 100.00a | 100.00a | 100.00a | 100.00a | 100.00a | 100.00a | 100.00a | 98.10 ± 0.95a |
| *Mean regardless of treatment ± SE | 45.00 ± 5.18e | 55.90 ± 5.90d | 61.60 ± 6.21d | 67.50 ± 6.72c | 69.40 ± 6.58bc | 72.50 ± 6.32abc | 75.00 ± 6.20ab | 76.90 ± 5.98a | |

*Note.* EM-PKOE = Emersense® AM 8025/palm kernel oil ester/water; BPH = Brown planthopper; Means followed by the same alphabet within columns (hour) are not significantly different by Tukey's test at $p = 0.05$. Means regardless of time (within column) and means regardless of treatment (within row) followed by the same alphabet are not significantly different by Tukey's test at $p = 0.05$

### $LC_{50}$ of 3rd and 4th Instar Nymphs of BPH in Emulsion Formulation

The $LC_{50}$ values were analyzed to determine the effective concentrations for the emulsion that caused 50% mortality of the nymphs' population at different exposure times, as shown in Table 7. Regardless of concentration, increased toxicity (lower $LC_{50}$ value) was recorded as the exposure time extended from 6 to 48 hr. At the earliest exposure time (6 hr), the $LC_{50}$ was estimated to occur at 1,061 ppm, similar to the result obtained in the non-formulated bioassay where at 1,000 ppm, 55% mortality was achieved. The $LC_{50}$ was reduced by half from the beginning of the observation until the final day of observation. Even though 48 hr showed the lowest $LC_{50}$ value (220 ppm), it was not significantly different with 24 hr (320 ppm), 30 hr (280 ppm), 36 hr (270 ppm), and 42 hr (260 ppm). As shown in Table 3, the $LC_{50}$ value for non-formulated *C. odorata* was 977 ppm on the final day of observation (72 hr of exposure), which was higher than the formulated *C. odorata* in EM-PKOE emulsion on the final day of observation (220 ppm after 48 hr of exposure). Evidently, incorporating 5% *C. odorata* into the EM-PKOE emulsion increased the extract's effectiveness in controlling BPH nymphs by 63.20%, similar to what was observed by Ezena et al. (2016), where the efficacy of *C. odorata* extract with the addition of sunflower oil, soap, roasted cocoa pods, red palm oil, coconut oil, sea salt, and shea butter in the formulation substantially increased in controlling diamondback moth (*Plutella xylostella* L.).

### Chemical Analysis of *C. odorata*

The GC-MS analysis of *C. odorata* leaf methanol extract recorded 65 compounds, and the identified components are presented in Table 8. The most abundant component in *C. odorata* was sesquiterpenes (24.14%), followed by fatty acid (14.65%), triterpenes (7.94%), flavonoids (6.05%), sterol (5.19%),

Table 7
*Variation of $LC_{50}$ (ppm) with respect to the duration of exposure of 3rd and 4th instar* Nilaparvata lugens *nymphs to the EM-PKOE emulsion formulated with* Chromolaena odorata *leaves extract*

| Exposure time (hr) | Slope ± SE | $LC_{50}$ (ppm) | 95% confidence limits (ppm) | Chi-square ($X^2$) |
|---|---|---|---|---|
| 6 | 1.18 ± 0.16 | 1,061a | 710 ~ 1,560 | 1.56 |
| 12 | 1.51 ± 0.19 | 530b | 370 ~ 740 | 1.73 |
| 18 | 1.78 ± 0.23 | 420bc | 300 ~ 560 | 0.51 |
| 24 | 2.04 ± 0.28 | 320bcd | 230 ~ 430 | 1.03 |
| 30 | 1.88 ± 0.26 | 280cd | 200 ~ 380 | 1.17 |
| 36 | 1.95 ± 0.31 | 270cd | 170 ~ 380 | 0.54 |
| 42 | 2.38 ± 0.43 | 260cd | 160 ~ 360 | 0.05 |
| 48 | 2.31 ± 0.42 | 220d | 130 ~ 310 | 0.56 |

*Note.* $LC_{50}$ is the concentration required to result in a 50% effect. Values with the same alphabet within a column are not significantly different by Tukey's test at $p = 0.05$; EM-PKOE = Emersense® AM 8025/palm kernel oil ester/water

Table 8
*Chemical constituents identified in the leaves of* Chromolaena odorata *from methanol extract*

| Compound class** | Retention time* | Relative percentage | Compound name |
|---|---|---|---|
| Sesquiterpenes | 26.86 | 0.16 | α -Cubebene |
| | 28.07 | 1.04 | α -Cubebene |
| | 30.03 | 1.97 | (E)-Caryophyllene |
| | 30.43 | 0.78 | Germacrene-D |
| | 31.51 | 0.55 | α -Humulene |
| | 32.49 | 0.69 | γ -Cadinene |
| | 32.69 | 0.26 | Germacrene-D |
| | 33.50 | 0.23 | α -Muurolene |
| | 34.48 | 2.04 | δ -Cadinene |
| | 36.69 | 1.02 | 1-Methyl-6-(3-methylbuta-1,3-dienyl)-7-oxabicyclo[4.1.0]heptane |
| | 37.01 | 1.05 | Caryophyllene oxide |
| | 39.81 | 0.39 | Cadin-4-en-10-ol |
| | 46.54 | 2.81 | Neophytadiene |
| | 47.41 | 0.62 | Neophytadiene |
| | 48.03 | 0.93 | Neophytadiene |
| | 64.04 | 0.90 | (+)-Sativen |
| | 63.13 | 4.48 | Aromadendran ('1') |
| | 64.68 | 3.42 | (+)-aromadendrene |
| | 65.56 | 0.30 | 1-alpha-, 10-alpha-epoxy-Amorph-4-ene |
| | 66.14 | 0.50 | Palustrol |
| Fatty acids | 38.04 | 0.34 | 12-Oxabicyclo[9.1.0]dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R*,3E,7E,11R*)] |
| | 49.57 | 0.18 | Hexadecanoic acid, methyl ester |
| | 50.90 | 4.38 | n-Hexadecanoic acid |
| | 55.12 | 0.18 | Linoleic acid, methyl ester |
| | 55.32 | 0.24 | Methyl linolenate |
| | 56.42 | 2.68 | 9,12-Octadecadienoic acid (Z,Z) |
| | 56.66 | 4.76 | 9,12,15-Octadecadienoic acid (Z,Z) |
| | 57.26 | 0.46 | Octadecanoic acid |
| | 72.25 | 0.53 | 1-Heptacosanol |
| | 77.17 | 0.90 | Lignoceric alcohol |
| Triterpenes | 75.38 | 3.63 | Squalene |
| | 89.51 | 3.30 | Olean-12-en-3-one |
| | 90.95 | 0.33 | β-Amyrin |
| | 92.42 | 0.68 | Lupeol acetate |
| Flavanoids | 72.10 | 0.90 | 5-Hydroxy-4',7-dimethoxyflavanone |
| | 83.54 | 2.84 | Flavone, 4',5,6,7-tetramethoxy |
| | 84.41 | 2.31 | Flavone, 3,4',5-trihydroxy-3',7-dimethoxy |

Table 8 *(continue)*

| Compound class** | Retention time* | Relative percentage | Compound name |
|---|---|---|---|
| Sterol | 85.68 | 2.51 | Stigmasterol |
| | 87.13 | 2.68 | β-Sitosterol |
| Diterpenes | 34.09 | 0.32 | γ -Amorphene |
| | 55.85 | 1.70 | Phytol |
| Vitamin E | 82.50 | 1.15 | dl-alpha-Tocopherol |
| Pyrrole | 3.48 | 0.19 | Methyl pyrrole |
| Unknown | 60.92 | 0.30 | 3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester |
| | 66.00 | 0.31 | Carbonic acid, 2-dimethylaminoethyl ethyl ester |
| | 75.71 | 0.83 | Ethyl 4-hydroxy-3-methoxyphenyl acetate |

*Note*.* = Retention time on the Rxi-5MS silica capillary column; ** = Search using Chemical Entities of Biological Interest (ChEBI) web-based application (Hastings et al., 2016)

diterpenes (2.02%), vitamin E (1.15%), and pyrrole (0.19%). Aromadendran ('1') (4.48%) was the main constituent of sesquiterpenes, followed by neophytadiene (4.36%), (+)-aromadendrane (3.42%), δ-cadinene (2.04%), (E)-caryophyllene (1.97%), α-cubebene (1.20%), caryophyllene oxide (1.05%), and germacrene-D (1.04%). As for fatty acid, the main constituent was 9,12,15-octadecadienoic acid (Z,Z) (4.76%), followed by *n*-hexadecanoic acid (4.38%), and 9,12-octadecadienoic acid (Z,Z) (2.68%). In triterpenes, the main components were squalene (3.63%) and olean-12-en-3-one (3.30%). Meanwhile, only stigmasterol (2.51%) was found to be sterol, while in flavonoid, the main components detected were flavone, 4',5,6,7-tetramethoxy (2.84%) and flavone, 3,4',5-trihydroxy-3',7-dimethoxy (2.31%). At the same time, phytol (1.70%) was the most abundant component found as a diterpenoid. Previously, Akinmoladun et al. (2007) found that the leaf extract of *C. odorata* contained terpenes, flavonoids and steroids. Jasnie (2009) and Joshi (2013) also observed the presence of germacrene-D, caryophyllene oxide, hexadecenoic acid, stigmasterol, γ -cadinene, δ -cadinene, and hexadecenoic acid in *C. odorata* plant extract.

Several studies on *C. odorata* extracts, which pose insecticidal properties, were also reported by other researchers against other insects. *Chromolaena odorata* extract effectively controlled the larval and pupal stages of the malaria vector (*Anopheles gambiae*) (Ileke & Olabimi, 2019). The presence of stigmasterol in *C. odorata* was identified to be responsible for the larvicidal activity in *Culex quinquefasciatus* and *Aedes aegypti*, in which the compounds that inhibit the acetylcholinesterase activity in the test insects possessed neurotoxicity action (Gade et al., 2017). Similar to this study, stigmasterol was also found in *C. odorata* methanol extract, possibly influencing BPH's mortality.

In addition, Langenheim (1994) also stated that plant terpenes were often reported as having anti-herbivore defenses. As an example, germacrene-D was previously studied to have deterrent effects against herbivores (Kiran & Devi, 2007), repellent activity against ticks (Birkett et al., 2008) and aphids (Bruce et al., 2005), as well as having insecticidal activity against mosquitoes (Langenheim, 1994). Another biological activity was also found in phytol (diterpene), which successfully controlled severe bacterial disease of ornamental fish, *Carassius auratus*, caused by *Bacillus licheniformis* (Saha & Bandyopadhyay, 2020), whereas squalene (triterpene) was responsible as an antioxidant (Huang et al., 2009) and antitumor (Huang et al., 2009; Senthilkumar et al., 2006).

## CONCLUSION

The solvent type significantly influenced the extraction yield, with methanol surpassing hexane by 69%. *Chromolaena odorata* methanol extract showed a greater mortality rate and a lower $LC_{50}$ value after 72 hr against the 3$^{rd}$ and 4$^{th}$ instar nymphs of BPH during the screening bioassay. An improved efficacy by 56% mortality was observed in the formulated bioassay when *C. odorata* methanol extract was successfully incorporated as the a.i. into EM-PKOE macroemulsion. Furthermore, insect mortality could also be attributed to the bioactive content of the leaf extract. *C. odorata* methanol extract was well known for its insecticidal effects against a variety of agricultural pests and diseases, especially with the presence of the terpenes group. Although macroemulsion showed a significant reduction in mortality and an increase in efficacy, formulation improvements are necessary as the world is increasingly moving towards nanoemulsion. Hence, exploration of the isolation and formulation of specific chemical compounds will enable the development of a new formulation of bio-insecticide for crop protection purposes.

## ACKNOWLEDGMENTS

The authors express their highest gratitude to Universiti Putra Malaysia for providing financial assistance through the Universiti Putra Malaysia Graduate Research Fellowship (GRF) and Putra Grant-Putra Graduate Initiative (GP-IPS/2018/9610300).

## REFERENCES

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, *18*(2), 265-267. https://doi.org/10.1093/jee/18.2.265a

Acero, L. H. (2014). Dried Siam weed (*Chromolaena odorata*) as rice weevils' (*Sitophilus oryza*) eradicant. *International Journal of Chemical Engineering and Applications*, *5*(5), 363-366. https://doi.org/10.7763/ijcea.2014.v5.410

Ahmed, M., Peiwen, Q., Gu, Z., Liu, Y., Sikandar, A., Hussain, D., Javeed, A., Shafi, J., Iqbal, M. F., An, R., Guo, H., Du, Y., Wang, W., Zhang, Y., & Ji, M. (2020). Insecticidal activity and biochemical composition of *Citrullus colocynthis*, *Cannabis indica*, and *Artemisia argyi* extracts against cabbage aphid (*Brevicoryne brassicae* L.). *Scientific Reports*, *10*, 522. https://doi.org/10.1038/s41598-019-57092-5

Akinmoladun, A. C., Ibukun, E. O., & Dan-Ologe, I. A. (2007). Phytochemical constituents and properties of extracts from the leaves of *Chromolaena odorata*. *Scientific Research and Essay*, *2*(6), 191–194.

Ali, E., Liao, X., Yang, P., Mao, K., Zhang, X., Shakeel, M., Salim, A. M. A., Wan, H., & Li, J. (2017). Sublethal effects of buprofezin on development and reproduction in the white-backed planthopper, *Sogatella furcifera* (Hemiptera: Delphacidae). *Scientific Reports*, *7*, 16913. https://doi.org/10.1038/s41598-017-17190-8

Al-Mansoub, M. A., Asmawi, M. Z., & Murugaiyah, V. (2014). Effect of extraction solvents and plant parts used on the antihyperlipidemic and antioxidant effects of *Garcinia atroviridis*: A comparative study. *Journal of the Science of Food and Agriculture*, *94*(8), 1552–1558. https://doi.org/10.1002/jsfa.6456

Appah, S., Jia, W., Ou, M., Wang, P., & Asante, E. A. (2020). Analysis of potential impaction and phytotoxicity of surfactant-plant surface interaction in pesticide application. *Crop Protection*, *127*, 104961. https://doi.org/10.1016/j.cropro.2019.104961

Aswathanarayan, J. B., & Vittal, R. R. (2019). Nanoemulsions and their potential applications in the food industry. *Frontiers in Sustainable Food Systems*, *3*, 95. https://doi.org/10.3389/fsufs.2019.00095

Awang, A. H. C. (2022, January 29). 1,000 petani di Yan kerugian akibat 'bena perang' [1,000 farmers in Yan lost due to brown planthopper]. *Astro Awani*. https://www.astroawani.com/berita-malaysia/1000-petani-di-yan-kerugian-akibat-bena-perang-344170

Azeem, A., Rizwan, M., Ahmad, F. J., Iqbal, Z., Khar, R. K., Aqil, M., & Talegaonkar, S. (2009). Nanoemulsion components screening and selection: A technical note. *AAPS PharmSciTech*, *10*, 69–76. https://doi.org/10.1208/s12249-008-9178-x

Azwanida, N. N. (2015). A review on the extraction methods used in medicinal plants, principles, strengths, and limitations. *Medicinal and Aromatic Plants*, *4*(3), 1000196. https://doi.org/10.4172/2167-0412.1000196

Balachiranjeevi, C. H., Prahalada, G. D., Mahender, A., Jamaloddin, Md., Sevilla, M. A. L., Marfori-Nazarea, C. M., Vinarao, R., Sushanto, U., Baehaki, S. E., Li, Z. K., & Ali, J. (2019). Identification of a novel locus, *BPH38*(t), conferring resistance to the brown planthopper (*Nilaparvata lugens* Stal.) using an early backcross population in rice (*Oryza sativa* L.). *Euphytica*, *215*, 185. https://doi.org/10.1007/s10681-019-2506-2

Begashaw, B., Mishra, B., Tsegaw, A., & Shewamene, Z. (2017). Methanol leaves extract of *Hibiscus micranthus* Linn exhibited antibacterial and wound healing activities. *BMC Complementary and Alternative Medicine*, *17*, 337. https://doi.org/10.1186/s12906-017-1841-x

Birkett, M. A., Abassi, S. A., Kröber, T., Chamberlain, K., Hooper, A. M., Guerin, P. M., Pettersson, J., Pickett, J. A., Slade, R., & Wadhams, L. J. (2008). Antiectoparasitic activity of the gum resin, gum haggar, from the East African plant, *Commiphora holtziana*. *Phytochemistry*, *69*(8), 1710–1715. https://doi.org/10.1016/j.phytochem.2008.02.017

Bouda, H., Tapondjou, L. A., Fontem, D. A., & Gumedzoe, M. Y. D. (2001). Effect of essential oils from leaves of *Ageratum conyzoides*, *Lantana camara*, and *Chromolaena odorata* on the mortality of *Sitophilus zeamais* (Coleoptera, Curculionidae). *Journal of Stored Products Research*, *37*(2), 103–109. https://doi.org/10.1016/S0022-474X(00)00011-4

Bruce, T. J. A., Birkett, M. A., Blande, J., Hooper, A. M., Martin, J. L., Khambay, B., Prosser,

I., Smart, L. E., & Wadhams, L. J. (2005). Response of economically important aphids to components of *Hemizygia petiolata* essential oil. *Pest Management Science*, *61*(11), 1115–1121. https://doi.org/10.1002/ps.1102

Chanana, G. D., & Sheth, B. B. (1995). Particle size reduction of emulsions by formulation design-II: Effect of oil and surfactant concentration. *PDA Journal of Pharmaceutical Science and Technology*, *49*(2), 71-76.

Choupanian, M., Omar, D., Basri, M., & Asib, N. (2017). Preparation and characterization of neem oil nanoemulsion formulations against *Sitophilus oryzae* and *Tribolium castaneum* adults. *Journal of Pesticide Science*, *42*(4), 158-165. https://doi.org/10.1584/jpestics.D17-032

Danaei, M., Dehghankhold, M., Ataei, S., Davarani, F. H., Javanmard, R., Dokhani, A., Khorasani, S., & Mozafari, M. R. (2018). Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems. *Pharmaceutics*, *10*(2), 57. https://doi.org/10.3390/pharmaceutics10020057

Duffus, J. H. (1993). Glossary for chemists of terms used in toxicology (IUPAC recommendations 1993). *Pure and Applied Chemistry*, *65*(9), 2003-2122. https://doi.org/10.1351/pac199365092003

Ezena, G. N., Akotsen-Mensah, C., & Fening, K. O. (2016). Exploiting the insecticidal potential of the invasive Siam weed, *Chromolaena odorata* L. (Asteraceae) in the management of the major pests of cabbage and their natural enemies in southern Ghana. *Advances in Crop Science and Technology*, *4*(4), 1000230. https://doi.org/10.4172/2329-8863.1000230

Foliadi, J., Hambali, E., & Fujita, H. (2018). Study on surfactant-solvent mixture formulation and its application on pesticide emulsion product. In *IOP Conference Series*: *Earth and Environmental Science* (Vol. 209, No. 1, p. 012037). IOP Publishing. https://doi.org/10.1088/1755-1315/209/1/012037

Food and Agriculture Organization of the United Nations and World Health Organization. (2010). *Manual on development and use of FAO and WHO specifications for pesticides* (1st ed.). FAO and WHO.

Fotsing, Y. S. F., Kezetas, J. J. B., Batiha, G. E.-S., Ali, I., & Ndjakou, B. L. (2021). Extraction of bioactive compounds from medicinal plants and herbs. In H. A. El-Shemy (Ed.), *Natural medicinal plants*. IntechOpen. https://doi.org/10.5772/intechopen.98602

Gade, S., Rajamanikyam, M., Vadlapudi, V., Nukala, K. M., Aluvala, R., Giddigari, C., Karanam, N. J., Barua, N. C., Pandey, R., Upadhyayula, V. S. V., Sripadi, P., Amanchy, R., & Upadhyayula, S. M. (2017). Acetylcholinesterase inhibitory activity of stigmasterol and hexacosanol is responsible for larvicidal and repellent properties of *Chromolaena odorata*. *Biochimica et Biophysica Acta - General Subjects*, *1861*(3), 541–550. https://doi.org/10.1016/J.BBAGEN.2016.11.044

Garrood, W. T., Zimmer, C. T., Gorman, K. J., Nauen, R., Bass, C., & Davies, T. G. E. (2016). Field-evolved resistance to imidacloprid and ethiprole in populations of brown planthopper *Nilaparvata lugens* collected from across South and East Asia. *Pest Management Science*, *72*(1), 140-149. https://doi.org/10.1002/ps.3980

Gbolade, A. A., & Adebayo, T. A. (1993). Fumigant effects of some volatile oils on fecundity and adult emergence of *Callosobruchus maculatus* F. *International Journal of Tropical Insect Science*, *14*, 631–636. https://doi.org/10.1017/S174275840001804X

Han, F., Li, S., Yin, R., Liu, H., & Xu, L. (2008). Effect of surfactants on the formation and characterization of a new type of colloidal drug delivery system: Nanostructured lipid carriers. *Colloids and Surfaces A: Physicochemical and*

*Engineering Aspects*, *315*(1-3), 210-216. https://doi.org/10.1016/j.colsurfa.2007.08.005

Hashim, R. (2021, January 19). 213.7 hektar sawah padi diserang bena perang [213.7 hectares of rice fields attacked by brown planthopper]. *Sinar Harian*. https://www.sinarharian.com.my/article/119880/EDISI/2137-hektar-sawah-padi-diserang-bena-perang

Hastings, J., Owen, G., Dekker, A., Ennis, M., Kale, N., Muthukrishnan, V., Turner, S., Swainston, N., Mendes, P., & Steinbeck, C. (2016). ChEBI in 2016: Improved services and an expanding collection of metabolites. *Nucleic Acids Research*, *44*(D1), D1214–D1219. https://doi.org/10.1093/nar/gkv1031

Hazra, D. K., & Purkait, A. (2019). Role of pesticide formulations for sustainable crop protection and environment management: A review. *Journal of Pharmacognosy and Phytochemistry*, *8*(2), 686–693.

Heong, K. L., Tan, K. H., Garcia, C. P. F., Liu, Z., & Lu, Z. (2013). *Research methods in toxicology and insecticide resistance monitoring of rice planthoppers* (2nd ed.). International Rice Research Institute. https://doi.org/10.13140/2.1.4699.5527

Huang, Z.-R., Lin, Y.-K., & Fang, J.-Y. (2009). Biological and pharmacological activities of squalene and related compounds: Potential uses in cosmetic dermatology. *Molecules*, *14*(1), 540–554. https://doi.org/10.3390/molecules14010540

Ileke, K. D., & Olabimi, I. O. (2019). Insecticidal activities of *Chromolaena odorata* and *Vernonia amygdalina* leaf extracts against *Anopheles gambiae* (Diptera: Culicidae). *International Journal of Tropical Diseases*, *2*(1), 018. https://doi.org/10.23937/IJTD-2017/1710018

Ingrid, D. T., Akwanjoh, S. R., & Yacouba, M. (2020). Insecticidal activity of *Ageratum conyzoides* (Asteraceae) aqueous extracts against the grasshopper *Zonocerus variegatus* (Orthoptera: Pyrgomorphidae). *Journal of Agriculture and Ecology Research International*, *21*(8), 29-36. https://doi.org/10.9734/jaeri/2020/v21i830159

Jasnie, F. H. (2009). *Biological activities and chemical constituents of* Chromolaena odorata *(L.) King & Robinson* [Unpublished Master's thesis]. Universiti Malaya.

Jena, K. K., & Kim, S.-M. (2010). Current status of brown planthopper (BPH) resistance and genetics. *Rice*, *3*, 161-171. https://doi.org/10.1007/s12284-010-9050-y

Joshi, R. K. (2013). Chemical composition of the essential oil of *Chromolaena odorata* (L.) R. M. King & H. Rob. roots from India. *Journal of Chemistry*, *2013*, 195057. https://doi.org/10.1155/2013/195057

Kadu, P. J., Kushare, S. S., Thacker, D. D., & Gattani, S. G. (2011). Enhancement of oral bioavailability of atorvastatin calcium by self-emulsifying drug delivery systems (SEDDS). *Pharmaceutical Development and Technology*, *16*(1), 65-74. https://doi.org/10.3109/10837450903499333

Khan, S., Taning, C. N. T., Bonneure, E., Mangelinckx, S., Smagghe, G., & Shah, M. M. (2017). Insecticidal activity of plant-derived extracts against different economically important pest insects. *Phytoparasitica*, *45*, 113–124. https://doi.org/10.1007/s12600-017-0569-y

Khazanah Research Institute. (2019). *The status of the paddy and rice industry in Malaysia*. Khazanah Research Institute. https://www.krinstitute.org/assets/contentMS/img/template/editor/20190409_RiceReport_Full%20Report_Final.pdf

Khoa, D. B., Thang, B. X., Liem, N. V., Holst, N., & Kristensen, M. (2018). Variation in susceptibility of eight insecticides in the brown planthopper *Nilaparvata lugens* in three regions of Vietnam 2015-2017. *PLOS One*, *13*(10), e0204962. https://doi.org/10.1371/journal.pone.0204962

Kiran, S. R., & Devi, P. S. (2007). Evaluation of mosquitocidal activity of essential oil and sesquiterpenes from leaves of *Chloroxylon swietenia* DC. *Parasitology Research*, *101*, 413–418. https://doi.org/10.1007/s00436-007-0485-z

Kumar, M. S., Rana, D., Rani, B. J., & Agale, S. (2017). Insecticidal activity of different *Ocimum* L. spp. extracts against brown planthopper, *Nilaparvata lugens*, (Stal.) (Delphacidae: Homoptera). *Journal of Entomology and Zoology Studies*, *5*(6), 2343-23348.

Kumar, N., Kumar, R., Shakil, N. A., Sarkar, D. J., & Chander, S. (2019). Evaluation of fipronil nanoformulations for effective management of brown plant hopper (*Nilaparvata lugens*) in rice. *International Journal of Pest Management*, *65*(1), 86–93. https://doi.org/10.1080/09670874.2018.1468046

Langenheim, J. H. (1994). Higher plant terpenoids: A phytocentric overview of their ecological roles. *Journal of Chemical Ecology*, *20*, 1223–1280. https://doi.org/10.1007/BF02059809

Lawal, O. A., Opoku, A. R., & Ogunwande, I. A. (2014). Phytoconstituents and insecticidal activity of different solvent leaf extracts of *Chromolaena odorata* L., against *Sitophilus zeamais* (Coleoptera: Curculionidae). *European Journal of Medicinal Plants*, *5*(3), 237-247. https://doi.org/10.9734/EJMP/2015/6739

Lu, G. W., & Gao, P. (2010). Emulsions and microemulsions for topical and transdermal drug delivery. In V. S. Kulkarni (Ed.), *Handbook of non-invasive drug delivery systems: Non-invasive and minimally-invasive drug delivery systems for pharmaceutical and personal care products* (pp. 59-94). William Andrew. https://doi.org/10.1016/B978-0-8155-2025-2.10003-4

Mahdi, E. S., Sakeena, M., Abdulkarim, M., Abdullah, G., Abdul Sattar, M. Z., & Noor, A. M. (2011). Effect of surfactant and surfactant blends on pseudoternary phase diagram behavior of newly synthesized palm kernel oil esters. *Drug Design, Development and Therapy*, *5*, 311–323. https://doi.org/10.2147/DDDT.S15698

Mansor, W. M. N. H. W. (2022, January 19). Petani berputih mata padi diserang bena perang [Farmers are dismayed by the infestation of their rice fields by brown planthoppers]. *Sinar Harian*. https://www.sinarharian.com.my/article/183708/edisi/petani-berputih-mata-padi-diserang-bena-perang

Marzuki, N. H. C., Wahab, R. A., & Hamid, M. A. (2019). An overview of nanoemulsion: Concepts of development and cosmeceutical applications. *Biotechnology and Biotechnological Equipment*, *33*(1), 779–797. https://doi.org/10.1080/13102818.2019.1620124

Masarudin, M. J., Cutts, S. M., Evison, B. J., Phillips, D. R., & Pigram, P. J. (2015). Factors determining the stability, size distribution, and cellular accumulation of small, monodisperse chitosan nanoparticles as candidate vectors for anticancer drug delivery: Application to the passive encapsulation of [$^{14}$C]-doxorubicin. *Nanotechnology, Science and Applications*, *8*, 67–80.

Matsumura, M., & Sanada-Morimura, S. (2010). Recent status of insecticide resistance in Asian rice planthoppers. *Japan Agricultural Research Quarterly*, *44*(3), 225–230. https://doi.org/10.6090/jarq.44.225

Matur, B. M., & Davou, B. J. (2007). Comparative larvicidal property of leaf extract of *Chromolaena odorata* L. (Compositae) and chlorpyrifos (organophosphorus compound) on Simulium larvae. *Biomed Environmental Science*, *4*, 313–316.

Motwani, S. K., Khar, R. K., Ahmad, F. J., & Chopra, S. (2006). Effect of solvent quality on determination of particle size and polydispersity of nanoparticles. *Journal of Experimental*

*Nanoscience*, *1*(3), 307-316. https://doi.org/10.1080/17458080600960002

Mudalige, T., Qu, H., Haute, D. V., Ansar, S. M., Paredes, A., & Ingle, T. (2019). Characterization of nanomaterials: Tools and challenges. In A. L. Rubio, M. J. F. Rovira, M. M. Sanz, & L. G. Gómez-Mascaraque (Eds.), *Nanomaterials for food applications* (pp. 313-353). Elsevier. https://doi.org/10.1016/B978-0-12-814130-4.00011-7

National Pesticide Information Center. (n.d.). *Pesticide formulations: Topic fact sheet*. http://npic.orst.edu/factsheets/formulations.html

Nauen, R. (n.d.). *Activities of the Insecticide Resistance Action Committee (IRAC): A brief introduction*. https://irac-online.org/content/uploads/IRAC_Overview_ICE_2012.pdf

Nobbmann, U. (2017). *Polydispersity - What does it mean for DLS and chromatography?* Malvern Panalytical. https://www.materials-talks.com/polydispersity-what-does-it-mean-for-dls-and-chromatography/#header

Nuryanti, S. S. P., Martono, E., Ratna, E. S., & Dadang. (2018). The bioactivities of selected Piperaceae and Asteraceae plant extracts against brown plant hopper (*Nilaparvata lugens* Stål.). *Journal of the International Society for Southeast Asian Agricultural Sciences*, *24*(2), 70-78.

Pauzi, S. S. A. (2021, January 21). Bena perang serang 79 hektar sawah padi [Brown planthopper attacks 79 hectares of rice fields]. *Harian Metro*. https://www.hmetro.com.my/mutakhir/2021/01/666537/bena-perang-serang-79-hektar-sawah-padi-metrotv

Prajapati, R., Roy., S., Mishra, S., Raza, S. K., & Thakur, L. K. (2014). Formulation development, standardization, and antimicrobial activity of *Ageratum conyzoides* extracts and their formulation. *International Journal of Pharmacy and Pharmaceutical Sciences*, *6*(Suppl. 2), 369–374.

Pratap, A. P., & Bhowmick, D. N. (2008). Pesticides as microemulsion formulations. *Journal of Dispersion Science and Technology*, *29*(9), 1325–1330. https://doi.org/10.1080/01932690701866815

Pulce, C., & Descotes, J. (1996). Household products. In J. Descotes (Ed.), *Human toxicology* (pp. 683–702). Elsevier Science. https://doi.org/10.1016/b978-044481557-6/50030-7

Saha, M., & Bandyopadhyay, P. K. (2020). *In vivo* and *in vitro* antimicrobial activity of phytol, a diterpene molecule, isolated and characterized from *Adhatoda vasica* Nees. (Acanthaceae), to control severe bacterial disease of ornamental fish, *Carassius auratus*, caused by *Bacillus licheniformis* $PKBMS_{16}$. *Microbial Pathogenesis*, *141*, 103977. https://doi.org/10.1016/j.micpath.2020.103977

Said-Al Ahl, H. A. H., Hikal, W. M., & Tkachenko, K. G. (2017). Essential oils with potential as insecticidal agents: A review. *International Journal of Environmental Planning and Management*, *3*(4), 23–33.

Saxena, R. C., & Khan, Z. R. (1985). Effect of neem oil on survival of *Nilaparvata lugens* (Homoptera: Delphacidae) and on grassy stunt and ragged stunt virus transmission. *Journal of Economic Entomology*, *78*(3), 647–651.

Senthilkumar, S., Devaki, T., Manohar, B. M., & Babu, M. S. (2006). Effect of squalene on cyclophosphamide-induced toxicity. *Clinica Chimica Acta*, *364*(1–2), 335–342. https://doi.org/10.1016/j.cca.2005.07.032

Senthil-Nathan, S., Choi, M. Y., Paik, C. H., Seo, H. Y., Kim, J. D., & Kang, S. M. (2007). The toxic effects of neem extract and azadirachtin on the brown planthopper, *Nilaparvata lugens* (Stål) (BPH) (Homoptera: Delphacidae). *Chemosphere*, *67*(1), 80–88. https://doi.org/10.1016/j.chemosphere.2006.09.045

Senthil-Nathan, S., Choi, M.-Y., Paik, C.-H., Seo, H.-Y., & Kalaivani, K. (2009). Toxicity and physiological effects of neem pesticides applied to rice on the *Nilaparvata lugens* Stål, the brown planthopper. *Ecotoxicology and Environmental Safety*, *72*(6), 1707–1713. https://doi.org/10.1016/j.ecoenv.2009.04.024

Shnoudeh, A. J., Hamad, I., Abdo, R. W., Qadumii, L., Jaber, A. Y., Surchi, H. S., Alkelany, S. Z. (2019). Synthesis, characterization, and applications of metal nanoparticles. In R. K. Tekade (Ed.). *Biomaterials and bionanotechnology* (pp. 527-612). Academic Press. https://doi.org/10.1016/B978-0-12-814427-5.00015-9

Sis, H., & Birinci, M. (2009). Effect of nonionic and ionic surfactants on zeta potential and dispersion properties of carbon black powders. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *341*(1-3), 60–67. https://doi.org/10.1016/j.colsurfa.2009.03.039

Sneddon, J., Masuram, S., & Richert, J. C. (2007). Gas chromatography-mass spectrometry - Basic principles, instrumentation, and selected applications for detection of organic compounds. *Analytical Letters*, *40*(6), 1003-1012. https://doi.org/10.1080/00032710701300648

Stevenson, P. C., Arnold, S. E. J., & Belmain, S. R. (2014). Pesticidal plants for stored product pests on small-holder farms in Africa. In D. Singh (Ed.). *Advances in plant biopesticides* (pp. 149-172). Springer. https://doi.org/10.1007/978-81-322-2006-0_9

Tambellini, N. P., Zaremberg, V., Turner, R. J., & Weljie, A. M. (2013). Evaluation of extraction protocols for simultaneous polar and non-polar yeast metabolite analysis using multivariate projection methods. *Metabolites*, *3*(3), 592–605. https://doi.org/10.3390/metabo3030592

Tiwari, P., Kumar, B., Kaur, M., Kaur, G., & Kaur, H. (2011). Phytochemical screening and extraction: A review. *Internationale Pharmaceutica Sciencia*, *1*(1), 98-106.

Udebuani, A. C., Abara, P. C., Obasi, K. O., & Okuh, S. U. (2015). Studies on the insecticidal properties of *Chromolaena odorata* (Asteraceae) against adult stage of *Periplaneta americana*. *Journal of Entomology and Zoology Studies*, *3*(1), 318–321.

Uma, M. S., & Kumar, A. R. V. (2009). Laboratory evaluation of some botanicals against diamond back moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae). *Pest Management in Horticultural Ecosystems*, *15*(1), 41–47.

Wang, Z., Wang, X., Cang, T., Zhao, X., Wu, S., Qi, P., Wang, X., Xu, X., & Wang Q. (2018). Positive effects of an oil adjuvant on efficacy, dissipation, and safety of pyrimethanil and boscalid on greenhouse strawberry. *Ecotoxicology and Environmental Safety*, *160*, 127-133. https://doi.org/10.1016/j.ecoenv.2018.04.064

Yusoff, S. F., Haron, F. F., Asib, N., Mohamed, M. T. M., & Ismail, S. I. (2021). Development of *Vernonia amygdalina* leaf extract emulsion formulations in controlling gray mold disease on tomato (*Lycopersicon esculentum* Mill.). *Agronomy*, *11*(2), 373. https://doi.org/10.3390/agronomy11020373

Zhang, Q.-W., Lin, L.-G., & Ye, W.-C. (2018). Techniques for extraction and isolation of natural products: A comprehensive review. *Chinese Medicine*, *13*, 20. https://doi.org/10.1186/s13020-018-0177-x

Zheng, H., Mao, L., Yang, J., Zhang, C., Miao, S., & Gao, Y. (2020). Effect of oil content and emulsifier type on the properties and antioxidant activity of sea buckthorn oil-in-water emulsions. *Journal of Food Quality*, *2020*, 1540925. https://doi.org/10.1155/2020/1540925

Pertanika J. Trop. Agric. Sci. 47 (4): 1473 - 1489 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# *Paederia foetida* Ameliorates Diabetic Cardiomyopathy in Rats Models by Suppressing Apoptosis

**Amrah Javaid[1], Norsuhana Omar[1]*, Rozaziana Ahmad[1], Anani Aila Mat Zin[2], Aminah Che Romli[1] and Rilwanu Isah Tsamiya[2]**

*[1]Department of Physiology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kota Bharu, Kelantan, Malaysia*

*[2]Department of Pathology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kota Bharu, Kelantan, Malaysia*

## ABSTRACT

Diabetes mellitus is one of the most prevalent global public health issues associated with a higher risk of cardiovascular diseases, contributing to morbidity and mortality. Research has demonstrated that elevated reactive oxygen species (ROS) generation in diabetes can trigger apoptosis, exacerbating diabetic cardiomyopathy (DCM). This study investigates the cardioprotective effects of *Paederia foetida* in rats' models of type 2 diabetes induced by a high-fat diet (HFD) and streptozotocin (STZ) treatment. The diabetic model was established in Sprague Dawley rats by intraperitoneal injection of streptozotocin (STZ, 40 mg/kg). Sprague Dawley rats were treated with varied concentrations of standardized extract of *P. foetida* (50 mg/kg and 100 mg/kg), administered orally once daily for four weeks. Standardized extract from *P. foetida* has a range of therapeutic potential, including anti-inflammatory, antioxidant, and anti-diabetic properties. The common metabolic disorder indices and myocardial apoptosis were investigated. The findings from this study demonstrated increased expression of Bcl-2 and decreased expression of Bcl-2 Associated X-protein BAX as indicated by IRS scoring in cardiomyocytes, suggesting that *P. foetida* has a significant protective effect on diabetic cardiomyopathy by decreasing apoptosis. Increased Bcl-2 and decreased BAX levels may be related to regulating oxidative stress and mitochondrial pathways involving myocardial apoptosis.

ARTICLE INFO

*Article history:*
Received: 28 December 2023
Accepted: 09 September 2024
Published: 19 November 2024

DOI: https://doi.org/10.47836/pjtas.47.4.24

*E-mail addresses*:
dramrahjavaid80@gmail.com (Amrah Javaid)
suhanakk@usm.my (Norsuhana Omar)
drroza@usm.my (Rozaziana Ahmad)
ailakb@usm.my (Anani Aila Mat Zin)
aminahr@usm.my (Aminah Che Romli)
ridhwan@usm.my (Rilwanu Isah Tsamiya)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

*P. foetida* extract could be a potential intervention for attenuating cardiomyopathy in diabetes mellitus.

*Keywords*: Diabetic cardiomyopathy, hyperglycemia, myocardial apoptosis, *Paederia foetida*, type 2 diabetes mellitus

## INTRODUCTION

Diabetes mellitus, a metabolic disorder, is one of the most prevalent global public health issues associated with a higher risk of cardiovascular diseases, contributing to morbidity and mortality (Punthakee et al., 2018; Zhao et al., 2018). The leading cause of death globally comprises 17.9 million deaths annually, and 31% of all deaths have been identified as cardiovascular disease (Lu et al., 2021). Diabetes type 1 and type 2 are heterogeneous diseases with a wide range of clinical presentations and disease progression (ElSayed et al., 2023). Insulin resistance and pancreatic β-cell dysfunction are the key factors contributing to developing and progressing type 2 diabetes mellitus (Nishimura et al., 2022). Inflammation in adipose tissues, brought on by immune cell infiltration around hypertrophied adipocytes, such as macrophages, primarily initiates and sustains insulin resistance (Fasshauer & Blüher, 2015). High-fat diet (HFD) consumption modifies the gut microbiota, causing endotoxemia and chronic inflammation in several tissues, including adipose tissue (Unamuno et al., 2018). As a compensation mechanism, obesity-related insulin resistance is frequently accompanied by a transient rise in insulin secretion and the number of pancreatic β-cells. However, diabetes is ultimately brought on by the attenuation of insulin secretion and the decline in β-cell mass that results from this compensation ceasing to be sustained over time (Cani et al., 2007). As a result of excessive production or insufficient clearance of mitochondrial reactive oxygen species (ROS) during persistent hyperglycemia, oxidative stress develops, which is a significant contributing factor to diabetic microangiopathy and a major contributor to diabetic cardiomyopathy (DCM) (Jia et al., 2018). Diabetic cardiomyopathy is a myocardial pathology unique to diabetic patients with persistent hyperglycemia and cardiac failure (Dillmann, 2019). Hence, elevated reactive oxygen species (ROS) and hyperglycemia in diabetic myocardial tissue are risk factors. Type 2 diabetes has been recognized as a substantial risk factor for cardiovascular diseases despite the unclear etiology of cardiovascular morbidity and mortality (Dunlay et al., 2019).

Cardiac failure unrelated to valvular heart disease, hypertension, or artery disease can arise in diabetic cardiomyopathy, a pathophysiological condition (Dillmann, 2019). According to previous research, chronic hyperglycemia affects the energy preferences of cardiomyocytes, increases the production of free radicals, and induces an oxidative stress-like condition (Kukidome et al., 2006). It may remodel cardiac structure, an essential indicator of diabetic cardiomyopathy. Myocardial interstitial, cardiomyocyte hypertrophy, elevated oxidative stress inflammation, perivascular fibrosis and apoptosis, and indicators are

the main pathological characteristics of diabetic cardiomyopathy (Saisho,2014). In addition to accelerating cardiac injury and initiating mitochondrial oxidative damage in diabetes mellitus, oxidative stress under hyperglycemic conditions lowers antioxidant capacity and can cause cell death through necrosis or apoptosis (Sangweni et al., 2021; Sun et al., 2020). Moreover, earlier research has demonstrated that elevated ROS generation in diabetes can trigger apoptosis, exacerbating diabetic cardiomyopathy (Bhatt et al., 2015; Ji et al., 2017). Apoptosis is primarily mediated by a family of cysteine proteases known as caspases. There are intrinsic (mitochondria-driven) and extrinsic caspase-mediated apoptosis pathways (receptor-mediated) (Lee & Pervaiz, 2007). Pro- and anti-apoptotic Bcl-2 proteins in the intrinsic pathway regulate the release of cytochrome C from mitochondria. Notably, the significant generation of superoxide ($O_2^-$) anion in mitochondria makes the membranes more susceptible to ROS. Consequently, the membrane is broken down by the sustained ROS attack, which leads to permeability, the release of cytochrome C into the cytoplasm, and, ultimately, the induction of apoptotic cell death (Orrenius, 2007; Ott et al., 2007). Cysteine-dependent aspartate-specific proteases (caspases), divided into initiator and effector caspases, are a family of protease enzymes that play a crucial role in apoptosis.

Initially, the effector caspases, including caspase 3, 6, and 7, are activated in a cascade after the initiator caspase, known as caspase 9, forms a compound with cytochrome C (Wen et al., 2022; Zou et al., 2021). Subsequently, chromatin condensation and oligonucleosomal DNA fragmentation are brought on by caspase 3 activating the caspase-activated DNase (CAD) enzyme (Ge et al., 2019). Moreover, the heart is damaged by activating the mitochondria-dependent apoptotic pathway, a direct consequence of oxidative stress and inflammation (Raish,2017). In the extrinsic pathway, the ligand binding to its death receptor engages an adaptor protein that procures and activates procaspase 8. As a result, FasL binds to Fas, resulting in the subsequent activation of the fas-associated death domain (FADD) and caspase-8. Upon ligating the cell surface death receptor(s), the receptor-mediated pathway is activated, initiating a downstream effector mechanism mediated by the caspase family of cysteine proteases (Lee & Pervaiz, 2007). The activation of caspase-3, essential for the induction of apoptotic cell death, is also caused by caspases-8 and caspase-9. Activating effector caspases like caspase 3 is necessary for apoptosis via a mitochondrial or non-mitochondrial pathway (Patar et al., 2018). However, a family of proteins known as B-cell lymphoma-2 (Bcl-2) that control the permeability of the membrane of mitochondria is known to modulate apoptosis. Members of this family include the anti-apoptotic (Bcl-x, Bcl-2, Bcl-XL, and Bcl-w) and pro-apoptotic (Bax, Bak, Bad, and Bim) proteins (Cory & Adams, 2002; Lee et al., 2020). Thus, inhibiting oxidative stress and apoptosis may prevent diabetic cardiomyopathy.

The search for more aggressive cardioprotective treatments remains challenging despite advancements in managing diabetic mellitus (Dunlay et al., 2019; Mordi et al., 2020; Snell-Bergeon & Maahs, 2015). Due to their extensive biological activities, bioactive phytochemicals are currently intensively studied (Lu et al., 2021). Medicinal plants have received considerably more attention as a source of biologically active substances, such as antioxidant, antihyperglycemic, and antihyperlipidemic agents. They play a significant role in identifying new counteractive drugs (Tang & Halliwell, 2010).

Similarly, a previous study has shown that *Paederia foetida* (Rubiaceae) exhibits good anti-diabetic properties and has suggested dl-α-tocopherol, stigmastanol, 2-hexyl-1-decanol, n-hexadecanoic acid and as the bioactive compounds present in the chloroform extract. Others are 2-nonadecanone, stigmast-4-en-3-one, 4,4-dimethyl-, cholest-8(14)-en-3-ol, (3β,5α)-, stigmasterol, 1-ethyl-1-tetradecyloxy-1-silacyclohexane, gamma-sitosterol, stigmast-7-en-3-ol, (3β,5α,24S)-, scopoletin, and α-monostearin. This plant is predominantly found in Asian countries (Tan et al., 2020). Research has extensively documented that *Paederia foetida* (PF) leaf extract exhibited considerable anti-diabetic, antihyperlipidemic, and antioxidant potential in mice models (Kumar et al., 2014). Antioxidant activity of the phenolic content of *Paederia foetida* stems from their role as free radical scavengers, which reduces oxidative stress (Osman et al., 2009). However, the anti-apoptotic effects of the plant on a diabetic heart have not been explained. Therefore, this study investigated the cardioprotective effects of *Peaderia foetida* in rats' models of type 2 diabetes induced by a high-fat diet (HFD) and streptozotocin (STZ) treatment.

## MATERIALS AND METHODS

### Extraction

Twigs of fresh *Paederia foetida* were air-dried and ground into powder. The powdered twigs of *Paederia foetida* (1.2 kg) were soaked and extracted by organic solvents, namely hexane (Fisher chemical, UK), chloroform (Thermofisher, India), and methanol (Honeywell Research Chemicals), using the cold maceration method. Briefly, the powdered plant material was soaked in solvent hexane in a conical flask for 72 hr. The Whatman filter paper was used to filter the hexane suspension. The filtrate was concentrated under reduced pressure using the rotary vacuum evaporator to get the crude extracts.

Similarly, the hexane solvent was substituted with chloroform and then methanol by applying the similar extraction method mentioned above. These steps were repeated three times for each solvent to optimize the yield. The active crude extract was freeze-dried and kept at -20°C until use.

### Animals

Sprague Male Dawley rats ($n = 48$), aged 10–12 weeks and of weight 190–220 g, were used for this study. The animals were acclimatized in a room with a temperature of 25 ± 2°C and a 12-hr day-light cycle for a week. They

were divided randomly into two groups: a normal control group fed a basal diet ($n$ = 8) and an obese group placed on a high-fat diet ($n$ = 40) for four weeks. The obese group was fed HFD to induce obesity. After four weeks, out of 40 obese rats, type 2 diabetes was induced in the 32 obese rats ($n$ = 32) following an overnight fast by intraperitoneal injection of streptozotocin (STZ, 40 mg/kg). In contrast, the normal control rats were injected with normal saline. Rats with more than 170 mg/dL of blood glucose were considered diabetic. After seven days, diabetic rats (n=32) were randomly further divided into four groups, each consisting of 8 treated with either distilled water, metformin, or varied concentrations of standardized extract of *Paederia foetida* (Table 1). The standardized extract and metformin were administered orally once daily for four weeks. All rats were fed *ad libitum* throughout the experiment. The rats used in the experiment were handled following the ethical approval of the Animal Ethics Committee of Universiti Sains Malaysia.

The characteristics of each grouping are as follows:

Group 1: Normal control (NC) (Healthy rats treated with distilled water daily)

Group 2: Obese (O) (High-fat diet rats treated with distilled water daily)

Group 3: Diabetic control (DC) (Diabetic rats treated with distilled water/day)

Group 4: Diabetic + Metformin (D+M) (Diabetic rats treated with 300 mg/kg/day Metformin)

Group 5: Diabetic + 50 mg Standardized extract *Paederia foetida* (D + 50 mgPF) (Diabetic rats treated with 50 mg/kg/day *Paederia foetida*)

Group 6: Diabetic + 100 mg Standardized extract *Paederia foetida* (D + 100 mg PF) (Diabetic rats treated with 100 mg/kg/day *Paederia foetida*)

## Immunohistochemistry and Examination of Apoptotic Markers

After 28 days, the rats fasted overnight and were euthanized by exsanguination under ketamine-xylazine [100-300 mg/kg] via intracardiac puncture. The heart was carefully harvested, preserved in 10% formaldehyde, and processed for fixation and paraffin embedding. Fixed tissues were rehydrated and deparaffinized. Immunohistochemistry (IHC) determines the anti-apoptotic markers B-cell lymphoma-2 and Bcl-2 Associated X-protein (Bcl-2 & BAX).

Table 1
*Diabetic rats' group*

| Number of rats (n) | Exposure/Treatment |
|---|---|
| 8 | Diabetic control rats treated only with distilled water daily |
| 8 | Diabetic rats treated with metformin at 300 mg/kg daily |
| 8 | Diabetic rats treated with a standardized extract of *Paederia foetida* at 50 mg/kg daily |
| 8 | Diabetic rats treated with a standardized extract of *Paederia foetida* at 100 mg/kg daily |

*Note*. Characteristics of diabetic rats' groups used in the experiment

### *Assessment of Bcl-2*

Positive control tissue for Bcl-2 consists of lymph nodes and tonsils. It was diluted 1:100, and the Leica Bond TM system was used to stain paraffin-embedded human lymph node tissue. Antigen retrieval was carried out by high pressure in a citrate buffer (pH 6) following dewaxing and hydration. The section (5 µm) was occluded for 30 minutes at room temperature with 10% normal goat serum. The primary antibody (1% BSA) had been incubated overnight at 4°C. A biotinylated secondary antibody identified and visualized the primary antibody by an SP system conjugated with HRP.

### *Assessment of BAX*

Human esophageal malignancy served as the positive control for BAX. A Leica Bond TM system was diluted 1:100 and stained on paraffin-embedded human oesophageal carcinoma tissue. Antigen retrieval was accomplished by high pressure in a citrate buffer (pH 6) following dewaxing and hydration. The section (5 µm) was occluded for 30 min at room temperature with 10% normal goat serum. The primary antibody (1% BSA) had been incubated overnight at 4°C. A biotinylated secondary antibody recognized and visualized the primary antibody by an SP system conjugated with HRP.

### *Scoring System for Reading IHC Slides*

Various methods are used to determine assessment criteria and associated scoring points. These are aligned with the experiment's scientific purpose and the characteristics of the IHC markers used. The reference point involves a percentage of positively stained cells and the observed staining intensity. The scoring system employed for interpreting the IHC results in this study is described below.

### *Immunoreactive Scoring (IRS) System*

The immunoreactive scoring system (IRS) gives a range of 0–12 as a product of multiplication between the positive cells proportion score (0–4) and the staining intensity score (0–3) (Table 2). IRS was utilized for expressing a variety of IHC markers (VEGF, BMP and its receptors, vWF and many others) in bone studies by (Koerdt et al., 2017). The sample under examination stains for IHC marker heterogeneously, then each staining intensity is scored independently, and the results are added. An example of such an approach is given by (Fedchenko & Reifenrath, 2014)when a specimen encompassed 50% of the tumor cells with moderate intensity (2 × 2 = 4), 25% of tumor cells with intense immunostaining (1 × 3 = 3), and 25% of cells with weak intensity (1 × 1 = 1), the score was 4 + 3 + 1 = 8.

Table 2
*Interpretation of immunoreactive scoring system (IRS) scoring*

| IRS scoring | Interpretation |
|---|---|
| 0-1 | Negative |
| 2-3 | Positive: Weak |
| 4-8 | Positive: Moderate |
| 9-12 | Positive: Strong |

### Statistical Analysis

All data were analyzed using the Statistical Package for Social Science (SPSS) for Windows software version 21.0 (SPSS, Chicago, USA). Qualitative data were expressed as percentages. Statistical analysis between groups was made using one-way analysis of variance (ANOVA), and variables were expressed as mean ± standard deviation. $P$ value < 0.05 was considered statistically significant.

## RESULTS

Glucose levels in group 1 and group 2 remained at the normal range throughout the experiment. The normal control group increased by 7.03% of blood glucose, while the Obese group increased by 11.01%. There was no significant difference in plasma glucose levels during the obesity induction period. Furthermore, glucose levels increased significantly ($p < 0.05$) in group 3 by 10.29% compared to groups 4, 5, and 6. However, treated diabetic mice showed decreased blood glucose levels than the diabetic control group (group 3). Treating diabetic rats (group 5) with *Paederia foetida* extract (50 mg/kg) significantly decreased blood glucose levels compared to groups 4 and 6. There was a 27.19% reduction in blood glucose levels in group 5, followed by 23.14% in group 4 (Table 3). Thus, the low extract dose seemed to be the most effective antihyperglycemic in this study.

### Interpretation of Bcl-2 Scoring

The IRS shown below (Table 4) of all groups predict the increased expression of Bcl-2 in group E and group F as indicated by IRS scores that show strong staining of cells with Bcl-2 that indicates the increased manifestation of Bcl-2 in groups of rats treated with standardized extract (Figure 1). It indicates that the dose given to group E rats has significantly decreased the apoptosis in rats with increased expression of positive cells with Bcl-2 cardiomyocytes, indicating the treatment has a positive impact on decreasing the apoptosis in streptozotocin-induced rats.

Table 3
*Plasma glucose levels and changes before and during interventions*

| Rats' group | Plasma glucose levels (nmol/L) | | |
|---|---|---|---|
| | Week 0 | Week 4 | Changes |
| Group 1: Normal healthy rats | $4.55 \pm 0.35^{a}$ | $4.87 \pm 0.26^{a}$ | $0.32 \pm 0.09^{a}$ (7.03%) |
| Group 2 : Obese rats | $5.72 \pm 0.49^{a}$ | $6.35 \pm 1.55^{a}$ | $0.63 \pm 1.06^{a}$ (11.01%) |
| Group 3: Diabetic control rats | $26.64 \pm 5.82^{b}$ | $29.38 \pm 2.43^{b}$ | $2.74 \pm 3.39^{a}$ (10.29%) |
| Group 4: Diabetic + Metformin | $21.48 \pm 1.99^{a}$ | $16.51 \pm 11.38^{b}$ | $-4.97 \pm 9.39^{c}$ (-23.14%) |
| Group 5: Diabetic + 50 mg/kg standardized extract of *Paederia foetida* | $13.68 \pm 6.70^{c}$ | $9.96 \pm 4.15^{a}$ | $-3.72 \pm 2.55^{b}$ (-27.19%) |
| Group 6: Diabetic + 100 mg/kg standardized extract of *Paederia foetida* | $16.14 \pm 4.07^{d}$ | $13.43 \pm 9.99^{a}$ | $-2.71 \pm 5.29^{c}$ (-16.79%) |

*Note*. All values were expressed as mean ± SD of 6 groups. Data with different superscripts (a, b, c, and d) in the same column were considered significantly ($p < 0.05$) different

Table 4
*Immunoreactive scoring system (IRS) scoring for B-cell lymphoma 2 (Bcl-2)*

| Groups | | % Positive cells | Staining intensity | IRS scoring |
|---|---|---|---|---|
| Bcl2 - Control | | 3 | 4 | 12 |
| Group 1 | Normal Control | 3 | 2 | 6 |
| Group 2 | Obese | 2 | 2 | 4 |
| Group 3 | Diabetic control | 2 | 1 | 2 |
| Group 4 | Diabetes + metformin | 3 | 3 | 9 |
| Group 5 | Diabetes + 50 mg *Paederia foetida* | 3 | 2 | 6 |
| Group 6 | Diabetes + 100 mg *Paederia foetida* | 2 | 2 | 4 |

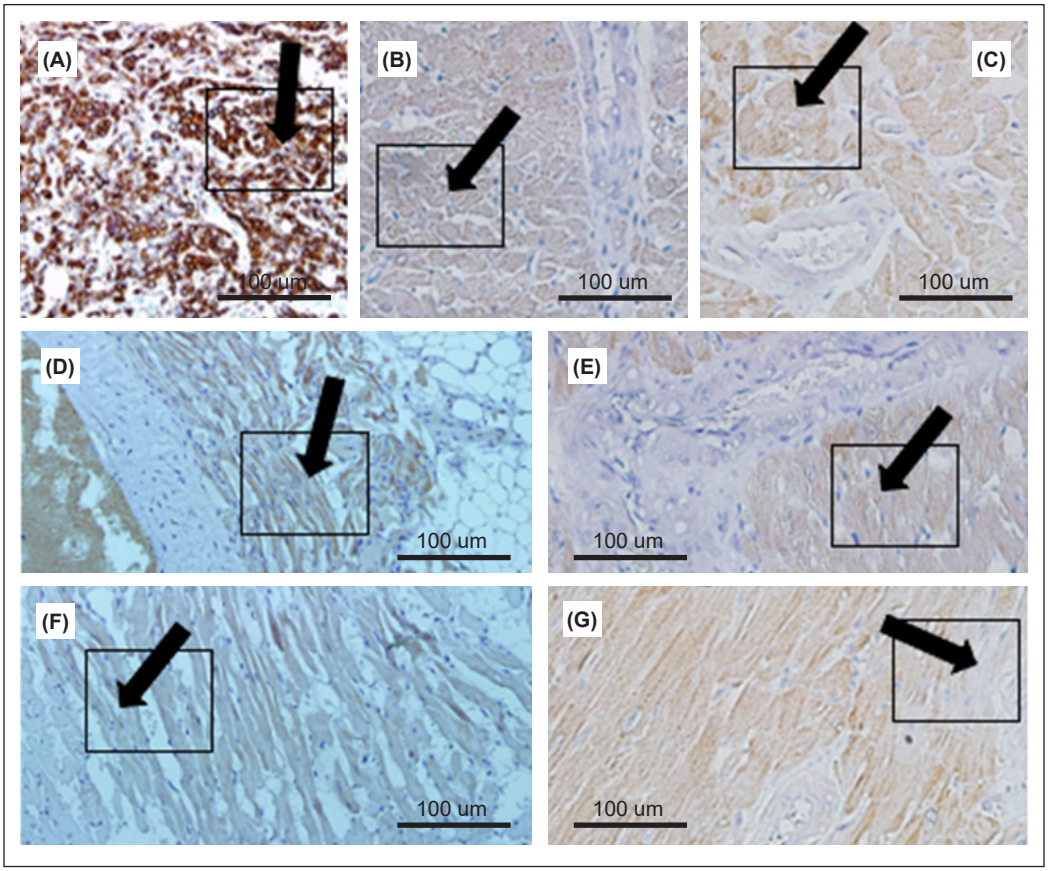



*Figure 1*. Representative photomicrograph of rats' cardiomyocytes with B-cell lymphoma 2 (Bcl-2)
*Note*. (A) Bcl-C = Cells of small figure (strong staining); (B) NC = Cells of small figure (weak staining); (C) O = Cells in small figure (strong staining); (D) DC = Cells in small figure (moderate staining); (E) D + M = Cells in small figure (moderate staining); (F) D + 50 mg PF = Cells of small figure (weak staining); (G) D + 100 mg PF = Cells of small figure (weak staining); Normal Control (NC); Obese (O); Diabetic control (DC); Diabetes + metformin (D + M); Diabetes + 50 mg *Paederia foetida* (D + 50 mg/kg PF); Diabetes + 100 mg *Paederia foetida* (D + 100 mg/kg PF)

Moreover, as identified by the immunoreactivity score (IRS), the increased expression of Bcl-2 in group 5 and group 6 indicated more staining of Bcl-2 cells as compared to the control, particularly in group 5, treated with a low dose (50 mg/kg) of standardized *Paederia foetida* extract.

It shows that the plant extract administered to these groups as an intervention has affected the expression of Bcl-2 in cardiomyocytes, indicating a positive impact on decreasing apoptosis in streptozotocin-induced diabetic rats, as shown by the IRS scoring (Figure 2).

## Interpretation of BAX Scoring

The IRS scores shown in Table 5 predicted the decreased expression of BAX in the group given 50 mg/kg *Paederia foetida* and the group given 100 mg/kg *Paederia foetida* as indicated by weak IRS scores that showed weak staining of cells with Bax as shown in Figure 3. It indicated the decreased expression of BAX in groups of rats treated with PF extract. It indicated that the 50 mg/kg PF extract given to groups of rats decreased the apoptosis in rats' cardiomyocytes as compared to 100 mg/kg PF as well as the diabetic control

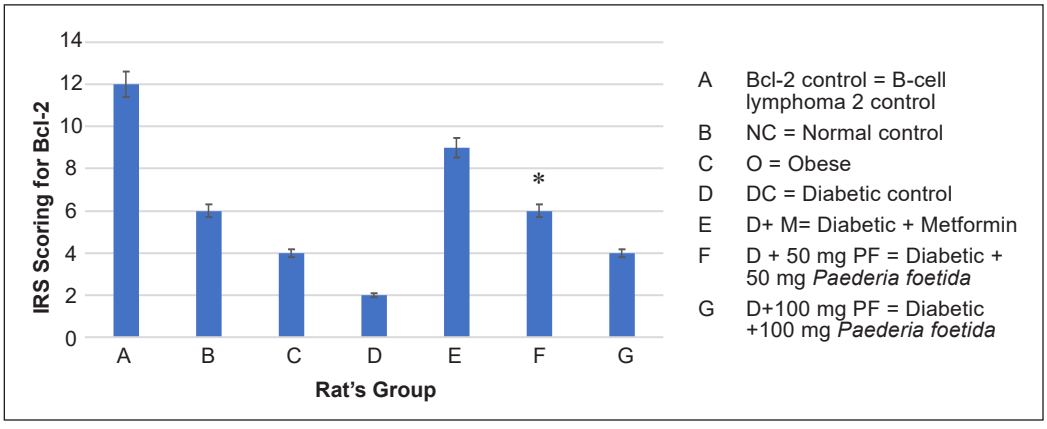



*Figure 2*. Immunoreactive scoring system (IRS) scoring for B-cell lymphoma 2 (Bcl-2) in rats' groups
*Note*. Group with * statistically significant ($p < 0.05$)

Table 5
*Immunoreactive scoring system (IRS) scoring for B-cell lymphoma 2 (Bcl-2)-associated X protein BAX in different groups of rats*

| Groups | | % Positive cells | Staining intensity | IRS scoring |
|---|---|---|---|---|
| BAX - Control | | 3 | 3 | 9 |
| Group 1 | Normal control | 3 | 2 | 6 |
| Group 2 | Obese | 3 | 3 | 9 |
| Group 3 | Diabetic control | 3 | 4 | 12 |
| Group 4 | Diabetes + Metformin | 2 | 3 | 6 |
| Group 5 | Diabetes + 50 mg PF | 3 | 1 | 3 |
| Group 6 | Diabetes + 100 mg PF | 2 | 2 | 4 |

*Note*: BAX control = Bcl-2 Associated X protein control

group, indicating that the treatment has a positive effect on decreasing the apoptosis in streptozotocin-induced diabetic rats (Figure 4).

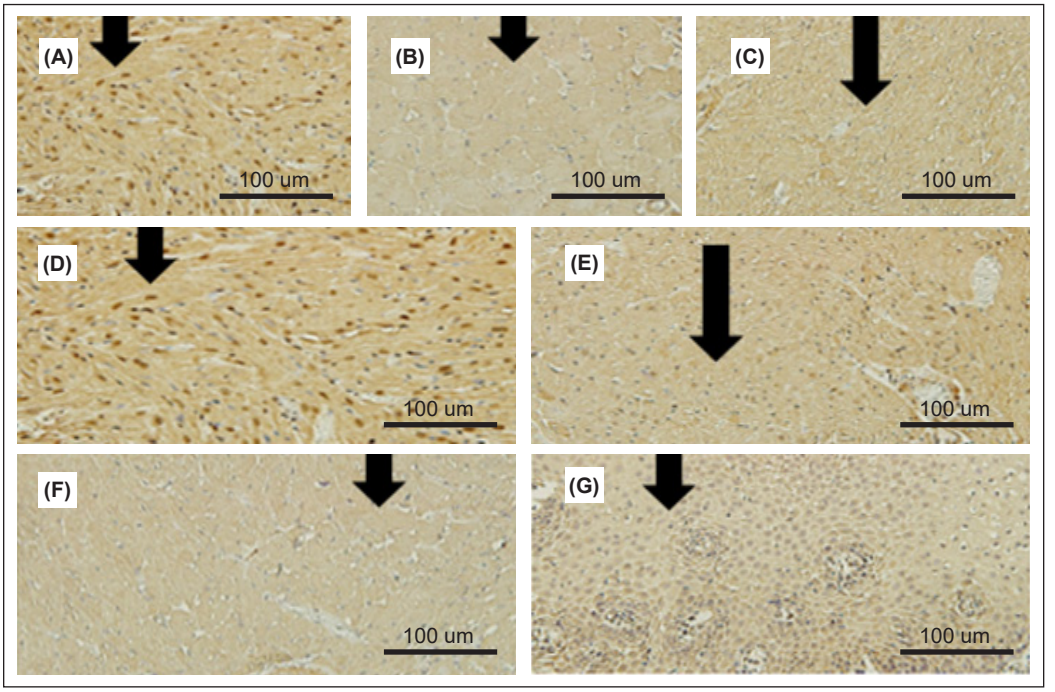



*Figure 3*. Representative photomicrograph of rat's cardiomyocytes with B-cell lymphoma 2 (Bcl-2)-associated X protein (BAX)
*Note*: (A) BAX-C = Cells of small figure (strong staining); (B) NC = Cells of small figure (weak staining); (C) O = Cells in small figure (strong staining); (D) DC = Cells in small figure (moderate staining); (E) D + M = Cells in small figure (moderate staining); (F) D + 50 mg PF = Cells of small figure (weak staining); (G) D + 100 mg PF = Cells of small figure (weak staining); Normal Control (NC); Obese (O); Diabetic control (DC); Diabetes + metformin (D + M); Diabetes + 50 mg *Paederia foetida* (D + 50 mg/kg PF); Diabetes + 100 mg *Paederia foetida* (D + 100 mg/kg PF)

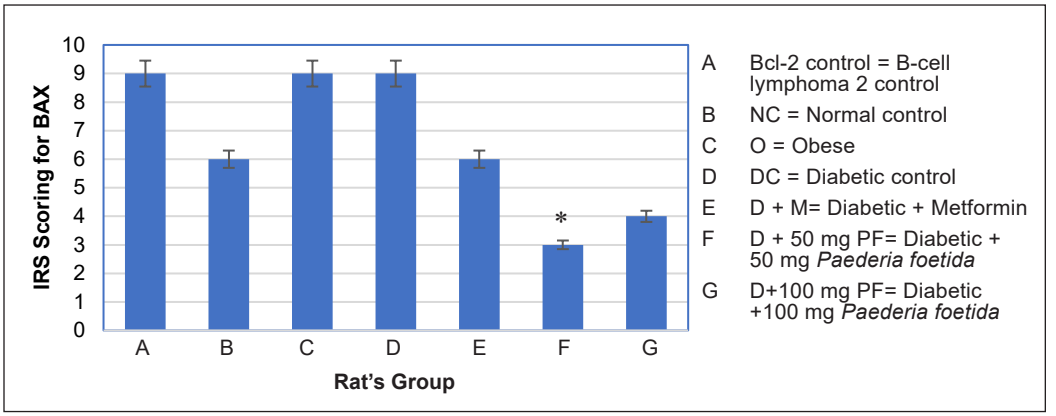



*Figure 4*. Immunoreactive scoring system (IRS) scoring for BAX in rats' groups
*Note*. Group with * considered significant ($p < 0.05$)

## DISCUSSION

This research observed a slight rise in the level of blood sugar in normal control rats. In obese rats, the level of blood glucose was mildly raised. The metabolic dysfunction induced by a high-fat diet (HFD) in rats is a valuable model for studying the pathogenesis of type 2 diabetes (Heydemann, 2016). Obesity develops due to increased calorie intake (Pang et al., 2013). Obesity causes white adipose tissue to become resistant to the antilipolytic effect of insulin and the amount of non-esterified fatty acids (NEFA) to increase. As a result, the ability of fat cells to bind fat decreases, and NEFA increases insulin resistance further in liver, muscle, and pancreatic beta-cells, along with impaired insulin secretion (Bays et al., 2004). Most chronic sequelae, such as cardiac tissue in interstitial metabolism disorders patients, impaired cellular function, fibrosis in vascular smooth muscle, and diminished contractile activity, are first caused by hyperglycemia after the onset of the disease (Shokoohi et al., 2019). However, the mice model treated with metformin and varied concentrations of *Paederia foetida* extract in this study showed a marked decrease in the level of blood glucose. Similarly, previous research on the extract from *Paederia foetida* demonstrated that the plant has a range of therapeutic potential, including anti-inflammatory, antioxidant, and anti-diabetic properties (Kumar et al., 2014).

In the present study, the diabetic model was established by Sprague Dawley mice injected with streptozotocin. Standardized *Peaderia foetida* extract treatment ameliorated pathological features of diabetic cardiomyopathy, including cardiomyocyte damage caused by oxidative stress and myocardial apoptosis. In addition, the main positive effect of intervention with *Peaderia foetida* in the diabetic model is likely through increased expression of Bcl-2 by cardiomyocytes and subsequent regulation of the anti-apoptotic pathway. In addition, the phenolic component of *Paederia foetida* has antioxidant activity and functions as a scavenger of free radicals, which decreases oxidative stress in diabetic conditions. Modulating oxidation-related pathways could protect vital organs such as the heart from diabetes-induced pathology. The histological hallmark of diabetic cardiomyopathy is increased myocardial apoptosis, associated with increased heart weight, decreased ventricular compliance, and heart failure. The current study observed that *Paederia foetida* treatment significantly prevents myocardial apoptosis in streptozotocin-induced type 2 diabetes in Sprague Dawley rats.

Furthermore, the damage to the myocardium in diabetes mellitus is broadly accepted to be caused by oxidative stress (Cai et al., 2002). Consequentially, hyperglycemia increases oxidative stress via mitochondrial ROS generation, accelerating cardiomyocyte apoptosis and cellular DNA damage and decreasing cardiac contractility, ultimately resulting in myocardial fibrosis(Aragno et al., 2006). Apoptosis is a primary mechanism of cell death characterized by a set of processes

that initiate a chain of molecular events culminating in cell death (Zhou et al., 2018). Persistent hyperglycemia has been linked to increased levels of reactive oxygen species (ROS) and diminished antioxidant defense efficiency, both of which can trigger signaling cascades leading to cell death (Wang et al., 2014; Zhang et al., 2016). The Bcl-2 protein family significantly controls the mitochondrial pathway of apoptosis. Antioxidant pathways in the endoplasmic reticulum, extracellular membrane, nucleus membrane, and inner membrane of the mitochondria can be listed as sites wherein Bcl-2 is found (Aslan et al., 2023).

*Paederia foetida* treatment in this study ameliorates Bcl-2 levels in cardiomyocytes, which mitigates apoptosis associated with diabetic cardiomyopathy. In response to apoptotic stimuli, Bcl-2 promotes survival by preventing the release of mitochondrial cytochrome C (Lv et al., 2012). After apoptotic stimuli, the ratio of BAX to Bcl-2 determines cell survival or demise (Emamaullee et al., 2006; Evans et al., 2002). It has been shown that the elevation of BAX expression and downregulation of Bcl-2 promotes cell death (Aravani et al., 2020). The higher levels of BAX and caspase-8 and a reduction of Bcl-2 in diabetic rats were demonstrated by their significant *P* value ($p < 0.05$) and IRS score (Fullstone et al., 2020).In the present research, the IRS scores of groups treated with *Paederia foetida* extract predicted the reduced expression of BAX and caspase-8 in both groups, as indicated by feeble staining of cells in both groups with low IRS scores more with 50 mg/kg PF dose as compared to 100 mg/kg PF possibly by reducing the endothelial dysfunction and reducing the β-cell apoptosis in diabetic rats. It indicated that the 50 mg/kg PF extract administered to rats decreased cardiomyocyte apoptosis, indicating that the treatment had a positive effect on reducing apoptosis in STZ-induced rats. This corresponds to a study examining the effect of the methanolic extract of *Paederia foetida* on prostate cancer cells (Pavlou & Kirmizis, 2016).

## CONCLUSION

This study suggests that *Paederia foetida* treatment ameliorates diabetes-associated-cardiomyopathy, as evidenced by its improvement of mitochondrial function via increasing Bcl-2 levels and decreasing BAX levels, which could subsequently inhibit myocardial apoptosis. This research demonstrated that standardized *Paederia foetida* extract could be a potential intervention for attenuating cardiomyopathy in diabetes mellitus.

## ACKNOWLEDGEMENTS

This work was supported by the USM Graduate Development Incentive Grant (311.PPSP.4404820). All authors contributed to conceptualizing, writing, and reviewing the manuscript.

## REFERENCES

Aragno, M., Mastrocola, R., Medana, C., Catalano, M. G., Vercellinatto, I., Danni, O., & Boccuzzi, G. (2006). Oxidative stress-dependent impairment of cardiac-specific transcription factors in

experimental diabetes. *Endocrinology*, *147*(12), 5967–5974. https://doi.org/10.1210/EN.2006-0728

Aravani, D., Foote, K., Figg, N., Finigan, A., Uryga, A., Clarke, M., & Bennett, M. (2020). Cytokine regulation of apoptosis-induced apoptosis and apoptosis-induced cell proliferation in vascular smooth muscle cells. *Apoptosis*, *25*(9), 648. https://doi.org/10.1007/S10495-020-01622-4

Aslan, A., Beyaz, S., Gok, O., Parlak, G., Can, M. I., Agca, C. A., Ozercan, I. H., & Parlak, A. E. (2023). Royal jelly protects brain tissue against fluoride-induced damage by activating Bcl-2/NF-κB/caspase-3/caspase-6/Bax and Erk signaling pathways in rats. *Environmental Science and Pollution Research, 30*(17), 49014-49025. https://doi.org/10.1007/s11356-023-25636-y

Bays, H., Mandarino, L., & DeFronzo, R. A. (2004). Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: Peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. *The Journal of Clinical Endocrinology and Metabolism*, *89*(2), 463–478. https://doi.org/10.1210/jc.2003-030723

Bhatt, N. M., Aon, M. A., Tocchetti, C. G., Shen, X., Dey, S., Ramirez-Correa, G., O'Rourke, B., Gao, W. D., & Cortassa, S. (2015). Restoring redox balance enhances contractility in heart trabeculae from type 2 diabetic rats exposed to high glucose. *American Journal of Physiology-Heart and Circulatory Physiology*, *308*(4), H291–H302. https://doi.org/10.1152/ajpheart.00378.2014

Cai, L., Li, W., Wang, G., Guo, L., Jiang, Y., & Kang, Y. J. (2002). Hyperglycemia-induced apoptosis in mouse myocardium. *Diabetes*, *51*(6), 1938–1948. https://doi.org/10.2337/diabetes.51.6.1938

Cani, P. D., Amar, J., Iglesias, M. A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A. M., Fava, F., Tuohy, K. M., Chabo, C., Waget, A., Delmée, E., Cousin, B., Sulpice, T., Chamontin, B., Ferrières, J., Tanti, J. F., Gibson, G. R., Casteilla, L., … Burcelin, R. (2007). Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes*, *56*(7), 1761–1772. https://doi.org/10.2337/db06-1491

Cory, S., & Adams, J. M. (2002). The Bcl2 family: Regulators of the cellular life-or-death switch. *Nature Reviews Cancer*, *2*(9), 647–656. https://doi.org/10.1038/nrc883

De, S., Ravishankar, B., & Bhavsar, G. C. (1994). Investigation of the anti-inflammatory effects of *Paederia foetida*. *Journal of Ethnopharmacology*, *43*(1), 31–38. https://doi.org/10.1016/0378-8741(94)90113-9

Dillmann, W. H. (2019). Diabetic cardiomyopathy. *Circulation Research*, *124*(8), 1160–1162. https://doi.org/10.1161/CIRCRESAHA.118.314665

Dunlay, S. M., Givertz, M. M., Aguilar, D., Allen, L. A., Chan, M., Desai, A. S., Deswal, A., Dickson, V. V., Kosiborod, M. N., Lekavich, C. L., McCoy, R. G., Mentz, R. J., & Piña, I. L. (2019). Type 2 diabetes mellitus and heart failure: A scientific statement from the american heart association and the heart failure society of america: This statement does not represent an update of the 2017 ACC/AHA/HFSA heart failure guideline update. *Circulation*, *140*(7), e294-e324. https://doi.org/10.1161/CIR.0000000000000691

ElSayed, N. A., Aleppo, G., Aroda, V. R., Bannuru, R. R., Brown, F. M., Bruemmer, D., Collins, B. S., Hilliard, M. E., Isaacs, D., Johnson, E. L., Kahan, S., Khunti, K., Leon, J., Lyons, S. K., Perry, M. lou, Prahalad, P., Pratley, R. E., Seley, J. J., Stanton, R. C., & Gabbay, R. A. (2023). 2. Classification and diagnosis of diabetes: Standards of care in diabetes—2023. *Diabetes Care*, *46*(Supplement_1), S19–S40. https://doi.org/10.2337/dc23-S002

Emamaullee, J. A., Shapiro, A. M. J., Rajotte, R. V., Korbutt, G., & Elliott, J. F. (2006). Neonatal porcine islets exhibit natural resistance to

hypoxia-induced apoptosis. *Transplantation*, *82*(7), 945–952. https://doi.org/10.1097/01.TP.0000238677.00750.32

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., & Nagata, S. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature*, *391*(6662), 43–50. https://doi.org/10.1038/34112

Evans, J. L., Goldfine, I. D., Maddux, B. A., & Grodsky, G. M. (2002). Oxidative stress and stress-activated signaling pathways: A unifying hypothesis of type 2 diabetes. *Endocrine Reviews*, *23*(5), 599–622. https://doi.org/10.1210/ER.2001-0039

Fasshauer, M., & Blüher, M. (2015). Adipokines in health and disease. *Trends in Pharmacological Sciences*, *36*(7), 461–470. https://doi.org/10.1016/j.tips.2015.04.014

Fedchenko, N., & Reifenrath, J. (2014). Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue - A review. *Diagnostic Pathology*, *9*(1), 221. https://doi.org/10.1186/S13000-014-0221-9/TABLES/4

Fullstone, G., Bauer, T. L., Guttà, C., Salvucci, M., Prehn, J. H. M., & Rehm, M. (2020). The apoptosome molecular timer synergises with XIAP to suppress apoptosis execution and contributes to prognosticating survival in colorectal cancer. *Cell Death and Differentiation, 27*(10), 2828-2842. https://doi.org/10.1038/s41418-020-0545-9

Ge, Q., Zhao, L., Ren, X. M., Ye, P., & Hu, Z. Y. (2019). LCZ696, an angiotensin receptor-neprilysin inhibitor, ameliorates diabetic cardiomyopathy by inhibiting inflammation, oxidative stress and apoptosis. *Experimental Biology and Medicine, 244*(12), 1028-1039. https://doi.org/10.1177/1535370219861283

Heydemann, A. (2016). An overview of murine high fat diet as a model for type 2 diabetes mellitus. *Journal of Diabetes Research*, *2016*(1), 2902351. https://doi.org/10.1155/2016/2902351

Ji, L., Liu, F., Jing, Z., Huang, Q., Zhao, Y., Cao, H., Li, J., Yin, C., Xing, J., & Li, F. (2017). MICU1 alleviates diabetic cardiomyopathy through mitochondrial $Ca^{2+}$–dependent antioxidant response. *Diabetes*, *66*(6), 1586–1600. https://doi.org/10.2337/db16-1237

Jia, G., Whaley-Connell, A., & Sowers, J. R. (2018). Diabetic cardiomyopathy: A hyperglycaemia- and insulin-resistance-induced heart disease. *Diabetologia*, *61*(1), 21–28. https://doi.org/10.1007/s00125-017-4390-4

Koerdt, S., Tanner, N., Rommel, N., Rohleder, N. H., Stoeckelhuber, M., Wolff, K. D., & Kesting, M. R. (2017). An immunohistochemical study on the role of oxidative and nitrosative stress in irradiated skin. *Cells Tissues Organs*, *203*(1), 12-19. https://doi.org/10.1159/000447584

Kukidome, D., Nishikawa, T., Sonoda, K., Imoto, K., Fujisawa, K., Yano, M., Motoshima, H., Taguchi, T., Matsumura, T., & Araki, E. (2006). Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. *Diabetes*, *55*(1), 120–127. https://doi.org/10.2337/diabetes.55.01.06.db05-0943

Kumar, V., Anwar, F., Ahmed, D., Verma, A., Ahmed, A., Damanhouri, Z. A., Mishra, V., Ramteke, P. W., Bhatt, P. C., & Mujeeb, M. (2014). *Paederia foetida* Linn. leaf extract: An antihyperlipidemic, antihyperglycaemic and antioxidant activity. *BMC Complementary and Alternative Medicine*, *14*(1), 76. https://doi.org/10.1186/1472-6882-14-76

Lee, J. H., Mellado-Gil, J. M., Bahn, Y. J., Pathy, S. M., Zhang, Y. E., & Rane, S. G. (2020). Protection from β-cell apoptosis by inhibition of TGF-β/Smad3 signaling. *Cell Death & Disease,*

*11*(3), 184. https://doi.org/10.1038/s41419-020-2365-8

Lee, S. C., & Pervaiz, S. (2007). Apoptosis in the pathophysiology of diabetes mellitus. *The International Journal of Biochemistry and Cell Biology*, *39*(3), 497–504. https://doi.org/10.1016/j.biocel.2006.09.007

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., & Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. *Cell*, *91*(4), 479–489. https://doi.org/10.1016/S0092-8674(00)80434-1

Lu, Q., Zheng, R., Zhu, P., Bian, J., Liu, Z., & Du, J. (2021). Hinokinin alleviates high fat diet/streptozotocin-induced cardiac injury in mice through modulation in oxidative stress, inflammation and apoptosis. *Biomedicine & Pharmacotherapy*, *137*, 111361. https://doi.org/10.1016/j.biopha.2021.111361

Lv, X., Yu, X., Wang, Y., Wang, F., Li, H., Wang, Y., Lu, D., Qi, R., & Wang, H. (2012). Berberine inhibits doxorubicin-triggered cardiomyocyte apoptosis via attenuating mitochondrial dysfunction and increasing Bcl-2 expression. *PLOS One*, *7*(10), e47351. https://doi.org/10.1371/journal.pone.0047351

Marles, R. J., & Farnsworth, N. R. (1995). Antidiabetic plants and their active constituents. *Phytomedicine*, *2*(2), 137–189. https://doi.org/10.1016/S0944-7113(11)80059-0

Mordi, N. A., Mordi, I. R., Singh, J. S., McCrimmon, R. J., Struthers, A. D., & Lang, C. C. (2020). Renal and cardiovascular effects of SGLT2 inhibition in combination with loop diuretics in patients with type 2 diabetes and chronic heart failure. *Circulation*, *142*(18), 1713–1724. https://doi.org/10.1161/CIRCULATIONAHA.120.048739

Nishimura, Y., Iwashita, M., Hayashi, M., Shinjo, T., Watanabe, Y., Zeze, T., Yamashita, A., Fukuda, T., Sanui, T., Sano, T., Asano, T., & Nishimura, F. (2022). XAF1 overexpression exacerbates diabetes by promoting pancreatic β-cell apoptosis. *Acta Diabetologica*, *59*(10), 1275–1286. https://doi.org/10.1007/s00592-022-01930-y

Orrenius, S. (2007). Reactive oxygen species in mitochondria-mediated cell death. *Drug Metabolism Reviews*, *39*(2–3), 443–455. https://doi.org/10.1080/03602530701468516

Osman, H., Rahim, A., Isa, N., & Bakhir, N. (2009). Antioxidant activity and phenolic content of *Paederia foetida* and *Syzygium aqueum*. *Molecules*, *14*(3), 970–978. https://doi.org/10.3390/molecules14030970

Ott, M., Gogvadze, V., Orrenius, S., & Zhivotovsky, B. (2007). Mitochondria, oxidative stress and cell death. *Apoptosis*, *12*(5), 913–922. https://doi.org/10.1007/s10495-007-0756-2

Pang, J., Rhodes, D. H., Pini, M., Akasheh, R. T., Castellanos, K. J., Cabay, R. J., Cooper, D., Perretti, M., & Fantuzzi, G. (2013). Increased adiposity, dysregulated glucose metabolism and systemic inflammation in galectin-3 KO mice. *PLOS One*, *8*(2), e57915. https://doi.org/10.1371/journal.pone.0057915

Patar, A. K., Sharma, A., Syiem, D., & Bhan, S. (2018). Chlorophyllin supplementation modulates hyperglycemia-induced oxidative stress and apoptosis in liver of streptozotocin-administered mice. *BioFactors*, *44*(5), 418–430. https://doi.org/10.1002/biof.1438

Pavlou, D., & Kirmizis, A. (2016). Depletion of histone N-terminal-acetyltransferase Naa40 induces p53-independent apoptosis in colorectal cancer cells via the mitochondrial pathway. *Apoptosis: An International Journal on Programmed Cell Death*, *21*(3), 298–311. https://doi.org/10.1007/S10495-015-1207-0

Punthakee, Z., Goldenberg, R., & Katz, P. (2018). Definition, classification and diagnosis of

diabetes, prediabetes and metabolic syndrome. *Canadian Journal of Diabetes*, *42*(Suppl 1), S10–S15. https://doi.org/10.1016/J.JCJD.2017.10.003

Raish, M. (2017). Momordica charantia polysaccharides ameliorate oxidative stress, hyperlipidemia, inflammation, and apoptosis during myocardial infarction by inhibiting the NF-κB signaling pathway. *International Journal of Biological Macromolecules*, *97*, 544–551. https://doi.org/10.1016/j.ijbiomac.2017.01.074

Saisho, Y. (2014). Glycemic variability and oxidative stress: A link between diabetes and cardiovascular disease? *International Journal of Molecular Sciences*, *15*(10), 18381–18406. https://doi.org/10.3390/ijms151018381

Sangweni, N. F., Mosa, R. A., Dludla, P. v., Kappo, A. P., Opoku, A. R., Muller, C. J. F., & Johnson, R. (2021). The triterpene, methyl-3β-hydroxylanosta-9,24-dien-21-oate (RA3), attenuates high glucose-induced oxidative damage and apoptosis by improving energy metabolism. *Phytomedicine*, *85*, 153546. https://doi.org/10.1016/j.phymed.2021.153546

Shokoohi, M., Soltani, M., Abtahi-Eivary, S. H., Niazi, V., Poor, M. R., Ravaei, H., Salimnejad, R., Moghimian, M., & Shoorei, H. (2019). Effect of hydro–alcoholic extract of *Olea europaea* on apoptosis–related genes and oxidative stress in a rat model of torsion/detorsion–induced ovarian damage. *Asian Pacific Journal of Reproduction*, *8*(4), 148-156. https://doi.org/10.4103/2305-0500.262831

Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., Wang, H. G., Reed, J. C., Nicholson, D. W., Alnemri, E. S., Green, D. R., & Martin, S. J. (1999). Ordering the cytochrome c–initiated caspase cascade: Hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9–dependent manner. *Journal of Cell Biology*, *144*(2), 281–292. https://doi.org/10.1083/jcb.144.2.281

Snell-Bergeon, J. K., & Maahs, D. M. (2015). Elevated risk of mortality in type 1 diabetes mellitus. *Nature Reviews Endocrinology*, *11*(3), 136–138. https://doi.org/10.1038/nrendo.2014.245

Sun, H. J., Xiong, S. P., Wu, Z. Y., Cao, L., Zhu, M. Y., Moore, P. K., & Bian, J. S. (2020). Induction of caveolin-3/eNOS complex by nitroxyl (HNO) ameliorates diabetic cardiomyopathy. *Redox Biology*, *32*, 101493. https://doi.org/10.1016/j.redox.2020.101493

Tan, D. C., Quek, A., Kassim, N. K., Ismail, I. S., & Lee, J. J. (2020). Rapid quantification and validation of biomarker scopoletin in *Paederia foetida* by qNMR and UV–Vis for herbal preparation. *Molecules*, *25*(21), 5162. https://doi.org/10.3390/molecules25215162

Tang, S. Y., & Halliwell, B. (2010). Medicinal plants and antioxidants: What do we learn from cell culture and Caenorhabditis elegans studies? *Biochemical and Biophysical Research Communications, 394*(1), 1–5. https://doi.org/10.1016/J.BBRC.2010.02.137.

Unamuno, X., Gómez-Ambrosi, J., Rodríguez, A., Becerril, S., Frühbeck, G., & Catalán, V. (2018). Adipokine dysregulation and adipose tissue inflammation in human obesity. *European Journal of Clinical Investigation*, *48*(9), e12997. https://doi.org/10.1111/eci.12997

Upadhyaya, S. (2013). Screening of phytochemicals, nutritional status, antioxidant and antimicrobial activity of *Paederia foetida* Linn. from different localities of Assam, India. *Journal of Pharmacy Research*, *7*(1), 139–141. https://doi.org/10.1016/j.jopr.2013.01.015

Wang, W., Lu, Q., Zhang, J., Wang, B., Liu, X., An, F., Qin, W., Chen, X., Dong, W., Zhang, C., Zhang, Y., & Zhang, M. (2014). HMGB1 mediates hyperglycaemia-induced cardiomyocyte apoptosis via ERK/Ets-1 signalling pathway. *Journal of Cellular and Molecular Medicine*, *18*(11), 2311–2320. https://doi.org/10.1111/jcmm.12399

Wen, C., Liu, C., Li, Y., Xia, T., Zhang, X., Xue, S., & Olatunji, O. J. (2022). Ameliorative potentials of the ethanolic extract from *Lycium chinense* leaf extract against diabetic cardiomyopathy. Insight into oxido-inflammatory and apoptosis modulation. *Biomedicine & Pharmacotherapy, 154*, 113583. https://doi.org/10.1016/J.BIOPHA.2022.113583.

Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B., & Korsmeyer, S. J. (1995). Bad, a heterodimeric partner for Bcl-xL and Bcl-2, displaces bax and promotes cell death. *Cell*, *80*(2), 285–291. https://doi.org/10.1016/0092-8674(95)90411-5

Zhang, F., Lin, X., Yu, L., Li, W., Qian, D., Cheng, P., He, L., Yang, H., & Zhang, C. (2016). Low-dose radiation prevents type 1 diabetes-induced cardiomyopathy via activation of AKT mediated anti-apoptotic and anti-oxidant effects. *Journal of Cellular and Molecular Medicine*, *20*(7), 1352–1366. https://doi.org/10.1111/jcmm.12823

Zhao, K., Yang, S. S., Wang, H. Bin, Chen, K., Lu, Z. H., & Mu, Y. M. (2018). Association between the hypertriglyceridemic waist phenotype and prediabetes in Chinese adults aged 40 years and older. *Journal of Diabetes Research*, *2018*(1), Article 1031939. https://doi.org/10.1155/2018/1031939

Zhou, H., Sun, Y., Zhang, L., Kang, W., Li, N., & Li, Y. (2018). The RhoA/ROCK pathway mediates high glucose-induced cardiomyocyte apoptosis via oxidative stress, JNK, and p38MAPK pathways. *Diabetes/Metabolism Research and Reviews*, *34*(6), e3022. https://doi.org/10.1002/dmrr.3022

Zou, J., Sui, D., Fu, W., Li, Y., Yu, P., Yu, X., & Xu, H. (2021). Total flavonoids extracted from the leaves of *Murraya paniculata* (L.) Jack alleviate oxidative stress, inflammation and apoptosis in a rat model of diabetic cardiomyopathy. *Journal of Functional Foods, 76*, 104319. https://doi.org/10.1016/J.JFF.2020.104319

Pertanika J. Trop. Agric. Sci. 47 (4): 1491 - 1509 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

*Review Article*

# Bee Propolis: Nature's Remedy for Bone Healing – A Narrative Review

**Jie Min Chai[1,2], Zurairah Berahim[1]*, Haslina Taib[1] and Wan Nazatul Shima Shahidan[1]**

*[1]School of Dental Sciences, Health Campus, Universiti Sains Malaysia,16150 Kubang Kerian, Kelantan, Malaysia*
*[2]Klinik Pergigian Tanjung Sepat d/a Klinik Kesihatan Tanjung Sepat, 42800 Tanjung Sepat, Selangor, Malaysia*

## ABSTRACT

Propolis is a resin-like compound bees produce from botanical substances mixed with their saliva and beeswax. It contains antioxidants like flavonoids and phenolic acids that promote bone healing. The promising potential of propolis in supporting bone healing has significant implications in various medical and dental fields, such as orthopedics, periodontology, orthodontics, and oral and maxillofacial surgery. This review aims to evaluate the existing body of research on the impact of propolis on bone healing. A comprehensive literature search spanning the last two decades until 2024 was conducted across reputable databases utilizing the search terms "propolis AND bone AND alveolar bone AND healing. Articles with these keywords, published in English and accessible from reputable databases like PubMed, Cochrane Library, Scopus, and Google Scholar, were included. Articles from unreliable sources, non-English publications, those without full-text access, and review articles or letters to editors were excluded. Initially, 1,974 articles were identified, and after removing duplicates and applying the inclusion and exclusion criteria, 54 articles were selected, and 31 were deemed relevant for the review. The literature indicates that propolis offers significant advantages in halting the progression of bone loss and expediting bone formation and maturation, primarily due to its antioxidant and anti-inflammatory properties. Consequently, incorporating propolis could be an effective and cost-efficient strategy for managing bone defects.

ARTICLE INFO

*Article history:*
Received: 28 December 2023
Accepted: 09 September 2024
Published: 19 November 2024

DOI: https://doi.org/10.47836/pjtas.47.4.25

*E-mail addresses*:
zurairah@usm.my (Zurairah Berahim)
jieminchai@hotmail.com (Jie Min Chai)
haslinakk@usm.my (Haslina Taib)
shima@usm.my (Wan Nazatul Shima Shahidan)
* Corresponding author

*Keywords*: Alveolar bone, bone healing, propolis

ISSN: 1511-3701
e-ISSN: 2231-8542

## INTRODUCTION

Bee products, including honey, royal jelly, beeswax, pollen, and propolis, have long been revered for their therapeutic potential. Propolis, also known as 'bee glue,' is a resinous mixture meticulously crafted by the bees from botanical sources. Its historical applications span ancient Egypt's embalming practices to wound care during the Anglo-Boer War and World War II. Propolis has been widely employed in traditional medicine across various medical fields (Kuropatnicki et al., 2013).

Like honey, the chemical composition of propolis is intricate, with variations that depend on its region's origin. This mixture predominantly contains resins, waxes, essential oils, pollen, and various organic compounds, including flavonoids and phenolic acids (Zabaiou et al., 2017). Additionally, it may contain antibiotics, enzymes, vitamins, and trace elements, all contributing to its remarkable biological properties, such as anti-inflammatory, antioxidant, antimicrobial, antiviral, anticancer, antibiotic, antifungal, and antiseptic effects (Lotfy, 2006; Marcucci, 1995; Orsolic & Jembrek, 2022). One notable aspect of propolis is its rich antioxidant content, particularly polyphenols, which underpin its reputation as a potent antioxidant. Flavonoids and cinnamic acid derivatives, including compounds like caffeic acid phenyl ester and caffeic acid, contribute to their anti-inflammatory attributes (Almeida & Menezes, 2002).

Bone health depends on a delicate balance between osteoblast and osteoclast activities, with the equilibrium between oxidants and antioxidants playing a crucial role. Maintaining this balance is essential for overall bone integrity and strength. Bone healing is the biological process by which bone tissue regenerates and repairs itself after an injury or surgical intervention. It is a dynamic, multi-step process involving coordinating various cell types and signaling networks, progressing through distinct phases: Inflammation, repair, and remodeling (Steppe et al., 2023). For optimal bone healing, balancing oxidative stress and inflammation is crucial. Acute inflammation and controlled oxidative stress are beneficial for initiating and supporting bone repair. However, chronic inflammation and excessive oxidative stress can impair the healing process and lead to complications.

In bone healing, propolis, which is rich in antioxidants, neutralizes reactive oxygen species (ROS) and free radicals, thus reducing oxidative stress (El-Haskoury et al., 2021; Tolay et al., 2024). It also inhibits the production of inflammatory cytokines (e.g., TNF-α, IL-1β) and enzymes involved in inflammation (Zulhendri et al., 2022). Propolis supports overall health by mitigating oxidative stress and inflammation and may enhance healing in various conditions. This review aims to assess the potential bone-healing properties of propolis in the medical and dental field.

## MATERIALS AND METHODS

### Study Design and Search Strategy

This narrative review synthesized existing research by sourcing articles from PubMed,

Cochrane Library, Scopus, and Google Scholar. A comprehensive search strategy encompassed using specific keywords, including "propolis AND bone AND alveolar bone AND healing," to identify articles for inclusion in this study.

### Criteria for Inclusion and Exclusion

The inclusion criteria involved selecting articles that contained the specified keywords, were published in English, and were accessible from reputable databases such as PubMed, Cochrane Library, Scopus, and Google Scholar. Articles released from the year 2000 until August 2024 were taken into consideration. Conversely, articles from unreliable sources (e.g., blogs or personal websites), those published in languages other than English, those lacking accessible full-text versions, and review articles and letters to editors were intentionally excluded.

## RESULTS AND DISCUSSION

In our search across four databases (PubMed, Cochrane Library, Scopus, and Google Scholar) using the keywords "propolis AND bone AND alveolar bone AND healing," a total of 1,974 articles were initially identified. Following the removal of duplicates, 759 articles remained. The titles and abstracts of these 759 articles were screened, and those that were not in English, letters to editors, abstracts only, and review articles were eliminated. It reduced the number to a shortlist of 54 potentially relevant articles. After the full-text review, 31 original articles were deemed relevant for our review (Table 1). These articles spanned the years from 2008 to 2024, representing diverse research from various regions, with most studies conducted in the Middle East (48%), followed by Southeast Asia (36%), East Asia (7%), South America (6%), and America (3%), (Figure 1).

The high percentage of propolis studies from the Middle East can be attributed to the region's rich tradition of using natural products and traditional medicine, where propolis has long been valued for its medicinal properties. Among Middle Eastern countries, Türkiye is the most active in conducting this research. Türkiye's Mediterranean climate, which supports a wide variety of flora, promotes abundant bee activity. The Southeast Asian region, with Indonesia leading, shows the second-highest level of research activity. The

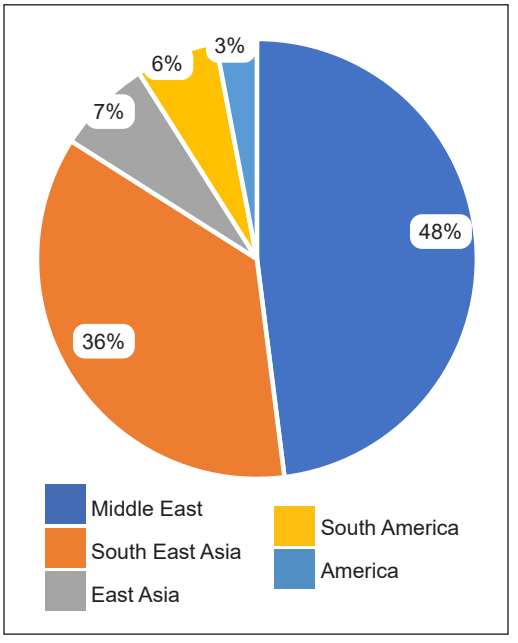



*Figure 1*. The pie chart illustrates the distribution of 31 studies from various regions

Table 1
*An overview of the studies included in this review*

| | References | Methodology | Results |
|---|---|---|---|
| 1 | Toker et al. (2008) | • The Wistar rats were randomly assigned to four sets: non-ligated, ligature only, ligature with a propolis dosage of 100 mg/kg body weight per day (Pro100), and ligature with a propolis dosage of 200 mg/kg body weight per day (Pro200).<br>• The ligatures were placed inside the gingival sulcus to induce a periodontitis model. On day 11 of the study, the rats were sacrificed for further analysis.<br>• Alveolar bone loss and histopathological evaluation were measured to assess the effects of the treatments. | • The ligature group exhibited significantly greater alveolar bone loss and osteoclast numbers compared to other groups.<br>• Both doses of propolis effectively reduced periodontitis-associated bone loss, with no variances detected between the two propolis groups. |
| 2 | Pileggi et al. (2009) | • Osteoclasts were induced from mouse marrow by culturing it in media containing 1,25-dihydroxyvitamin D3.<br>• Osteoclast-like cells were induced by culturing them in media containing Glutathione S-Transferase Receptor Activator of Nuclear Factor Kappa-B Ligand (GST-RANKL).<br>• These cells were then exposed to ethanol extracts of propolis or vehicle control at predetermined concentrations during the culture period.<br>• The assessment was performed using TRAP and actin ring assays. | • Propolis treatment resulted in a notable and dose-dependent decrease in multinuclear TRAP+ cells, indicating its efficacy in reducing osteoclast formation.<br>• Propolis decreased actin ring formation in osteoclast-like cells, suggesting its direct influence on osteoclast maturation. |
| 3 | Guney et al. (2011) | • This study involved 32 male Sprague-Dawley rats aged approximately 6 months, weighing between 280 and 490 g. Prior to their assignment to the groups, all rats experienced experimental femur fracture and fixation.<br>• The treatment groups were administered propolis orally at 200 milligrams per kg body weight per day for 3 or 6 weeks, while the control groups did not receive propolis.<br>• Radiological and biochemical evaluation, bone mineral density assessment and histopathological examination were conducted to evaluate the impact of propolis treatment on bone healing and regeneration. | • The study observed enhancements in bone healing, as well as reduced levels of antioxidant enzymes compared to the control group.<br>• The treatment group also exhibited time-dependent enhancements in both categories. |

Table 1 *(continue)*

| | References | Methodology | Results |
|---|---|---|---|
| 4 | Altan et al. (2013) | • Twenty-four male Wistar albino rats were grouped into the non-expansion group (control group), the expansion group only (OE), and the expansion with propolis group (PRO), which was given via orogastric tube. After a 5-day expansion phase, both the OE and PRO groups experienced twelve days of mechanical retention before being euthanized. Their upper jaws were isolated and processed for histological investigation.<br>• Histological examination was conducted to evaluate cells and new bone formation. | • The PRO group showed elevated inflammatory cell intensity, increased osteoblast count, and enhanced new bone formation compared to the others.<br>• The propolis group also showed increased bone-resorbing cells and newly formed blood vessels. |
| 5 | Aral et al. (2015) | • The rats were grouped into seven sets for the study: negative control (NC), periodontitis (P), diabetes (D), diabetes with periodontitis (DP), periodontitis with propolis treatment (P-Pro), diabetes with propolis treatment (D-Pro), and diabetes with periodontitis and propolis treatment (DP-Pro). Periodontitis and diabetes were induced by ligature placement and streptozocin injection, respectively.<br>• Blood samples were obtained to determine plasma cytokine and matrix metalloproteinases (MMP) levels, and histological analysis was conducted. | • The blood glucose levels of P, P-Pro, and D-Pro did not display significant variances compared to NC. Nevertheless, D, DP, and DP-Pro showed notable differences from NC.<br>• When comparing treatment and non-treatment groups, P-Pro demonstrated significantly lower alveolar bone loss.<br>• DP exhibited more significant bone loss compared to DP-Pro, while no statistically significant difference was observed between the D and D-Pro groups.<br>• The final measurements of the specific plasma component did not show any variations between groups. |
| 6 | Al-Molla et al. (2014) | • Forty New Zealand rabbits were grouped into four based on healing intervals of 1, 2, 4, and 6 weeks, with 10 animals in each group. Two bony holes were created in the tibia: one hole received a propolis-coated implant, while the second hole received an uncoated implant (control).<br>• Histological and immunohistochemical tests were conducted on all implants to detect the expression of osteocalcin and collagen type I. | • The histological analysis of titanium implants coated with propolis revealed an early osteogenic process compared to the control implants.<br>• Immunohistochemical findings demonstrated the presence of osteocalcin and collagen type I at the propolis-coated implants, signifying an accelerated osteogenic process. |

Table 1 *(continue)*

| | References | Methodology | Results |
|---|---|---|---|
| 7 | Bereket et al. (2014) | • Twenty-one rabbits were grouped into a control group that received water orally daily, a P100 group that received 100 mg/kg/d of propolis orally, and a P200 group that received 200 mg/kg/d of propolis orally. Following an osteotomy, the rabbits underwent distraction osteogenesis of the left mandible.<br>• Stereologic analyses were conducted to measure the connective tissue and new bone.<br>• Dual-energy X-ray measurements were performed 1 and 4 weeks after the procedure to evaluate hard tissue content and density. | • At four weeks, the X-ray results revealed higher hard tissue content and density in the propolis groups, with the P200 group showing the highest values.<br>• No significant differences were observed in connective tissue volume or the quantities of blood vessels among the groups. The volume of new bone was lowest in the P200 group. |
| 8 | Sherif et al. (2015) | • Twenty-four rats were grouped into 1. normal control, 2. diabetes + periodontitis, and 3. diabetes + periodontitis + propolis.<br>• An injection of streptozotocin-induced diabetes mellitus and blood glucose levels were measured. Periodontitis was induced by placing a ligature subgingivally on the first molar of the right mandibular molar. Propolis was given by gastric feeding at 400 mg/kg/day for 8 weeks.<br>• A scanning electron microscopic (SEM) examination was conducted to evaluate the integrity of the alveolar bone surface interproximal. | • Group 2 exhibited greater alveolar bone loss compared to the others.<br>• Specimens from groups 1 and 3 displayed a regular bone surface, whereas those from group 2 demonstrated an irregular and porous surface with extensive resorption. |
| 9 | Zohery et al. (2017) | • A split-mouth design was implemented using the premolars of three healthy Mongrel dogs. A Grade II furcation defect was surgically created. One side was treated with NanoBone graft, while the other was treated with propolis. Both sides were covered with a collagen membrane.<br>• The dogs were euthanized after 4 weeks, and the defective parts of the jawbone were processed for histological evaluation. | The percentage of bone fill and surface area in defects treated with propolis powder exceeded those in NanoBone-filled defects. |
| 10 | Aydin et al. (2018) | • Twenty-four rabbits were grouped into groups of 8. Implants were placed into the tibia in all groups. The control group was implant only, the local group had propolis applied to the slots before implant placement, and the systemic group received oral propolis solution daily after implantation. | • In both propolis groups, SOD activity showed an increase compared to the control group, though it was not statistically significant.<br>• MDA levels were significantly lower in both propolis groups than in control. |

Table 1 *(continue)*

| | References | Methodology | Results |
|---|---|---|---|
| | | • Biochemical tests were conducted before rabbit sacrifice on the 28th day, including assessing Vitamin D, calcium, phosphorus, and antioxidant enzyme values. Additionally, tests were conducted to determine malondialdehyde (MDA) levels, superoxide dismutase (SOD) activity, catalase activity, and glutathione peroxidase (GSH-Px) activity. | • A statistically significant increase was observed in the GSH level of the treated group compared to the control group.<br>• No significant differences were detected in calcium and phosphorus levels. However, vitamin D levels rose significantly in both local and systemic groups. |
| 11 | Somsanith et al. (2018) | • Titanium dioxide ($TiO_2$) nanotubes (TNT) were fabricated on commercially pure Ti (CP-Ti) plates, followed by propolis loading.<br>• In vitro investigations utilized untreated CP-Ti, TNT, and propolis-loaded TNT (PL-TNT-Ti) plates, assessing them through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, alkaline phosphatase (ALP) assay, crystal violet staining, and fluorescence microscopy.<br>• For *in vivo* studies, the rats were grouped into TNT implants and PL-TNT-Ti implants. They were observed for 1 and 4 weeks and evaluated via μ-CT analysis, histological staining, and immunoassay. | • The PL-TNT-Ti group demonstrated enhanced osteoblast cell proliferation and differentiation compared to other groups. μ-CT and Hematoxylin and Eosin (H&E) histological analysis after 1 and 4 weeks of implantation into rat mandibles revealed increased osteogenesis around the PL-TNT-Ti implant.<br>• The staining showed well-formed collagen for bone formation on the PL-TNT-Ti.<br>• The expression of BMP-2 and BMP-7 was increased around the PL-TNT-Ti, enhancing collagen fiber expression and osteogenic differentiation while decreasing inflammatory cytokines. |
| 12 | Meimandi-Parizi et al. (2018) | • This study created bone defects in healthy rats and randomly divided them into six groups of 12 rats: (1) autograft, (2) untreated, (3) chitosan, (4) demineralized bone matrix (DBM), (5) chitosan-propolis, and (6) DBM-propolis.<br>• On the $28^{th}$, $42^{nd}$, and $56^{th}$ postoperative days, clinical examinations were conducted alongside the evaluation of radiographic images to assess bone repair capabilities.<br>• Histopathological and biochemical evaluations were performed to analyze the outcomes further. | • The DBM-propolis group exhibited superior structural and biomechanical properties compared to others.<br>• In the chitosan and untreated groups, fibrous connective tissue primarily filled the defect site, while the autograft group predominantly showed cartilage with a smaller volume of fibrous bone.<br>• By the $56^{th}$ day after the injury, the DBM-propolis group displayed newly formed tissues comprised mainly of woven bone and hyaline cartilage. |
| 13 | Pereira et al. (2018) | • This study employed thin-layer chromatography and bioautography techniques to analyze propolis and determine the antibacterial assay against Gram-negative bacteria. | • The infected and treated with propolis group exhibited increased new bone tissue within alveoli, featuring bony trabeculae surrounding small cavities filled with loose connective tissue containing blood vessels. |

Table 1 *(continue)*

| | References | Methodology | Results |
|---|---|---|---|
| | | • *In vitro* cytotoxicity assays assessed the immunoregulatory activity of propolis on rat spleen leukocytes.<br>• For the animal model, 32 Wistar rats underwent maxillary first molar extractions, with the right socket immediately contaminated with lipopolysaccharide (LPS). After 14 days, rats were divided into pure propolis extract (EPP) treated and untreated groups.<br>• Histological and TRAP immunohistochemistry analyses were conducted on contaminated and uncontaminated alveolar bone samples to evaluate the effects of therapy on inflammation. | • The uncontaminated group treated with propolis showed bone cells along the periphery of the osteoid tissue trabeculae. It also contained fibrous connective tissue with numerous capillaries filling large cavities. |
| 14 | Zohery et al. (2018) | • A split-mouth design was employed in healthy mongrel dogs. Grade II furcation defects were created in mandibular premolars. One side was treated with nanohydroxyapatite graft, while the other was treated with propolis. Both sides were covered with a collagen membrane.<br>• The animals were sacrificed after one and three months for histological evaluation. | • After one month, the histological evaluation indicated new bone formation in both test groups.<br>• After three months, bone trabeculae appeared thinner in the nanohydroxyapatite group compared to the propolis group.<br>• Analysis revealed a notable increase in bone height and surface area in the propolis group compared to the nanohydroxyapatite group. |
| 15 | Wimolsantirungsri et al. (2018) | • Osteoclast precursors were isolated from peripheral blood and cultivated with different non-toxic concentrations of propolis extract.<br>• Osteoclast formation was assessed using TRAP staining, actin ring formation, and real-time polymerase chain reaction. Additionally, osteoclast function was evaluated through the resorption pit assay. | • Non-toxic concentrations of propolis extract demonstrated significant suppression of osteoclast formation by reducing the percentages of TRAP-positive multinuclear cells and the ratios of cells with F-actin ring formation in a dose-dependent manner.<br>• Propolis reduced the expression of several osteoclast-specific genes in a dose-dependent manner. |
| 16 | Ibrahim et al. (2019) | • Sixteen critical-sized furcation defects were created on the mandibular premolars of dogs, and they were then divided into three groups: Group I received propolis, Group II received nanohydroxyapatite graft, and Group III received a combination of propolis and nanohydroxyapatite graft.<br>• Histological analyses were conducted after one and three months. | • After one month, all three experimental groups exhibited early periodontal tissue regeneration characteristics, including cementum and newly formed bone with inserted periodontal ligament (PDL) fibers. The propolis group demonstrated the highest bone height and bone surface area.<br>• After three months, denser bone occupying a larger surface area of the furcation defect was observed, particularly in the propolis group. |

Table 1 *(continue)*

| | References | Methodology | Results |
|---|---|---|---|
| 17 | Lim et al. (2019) | • The experimental groups included a control group (1% DMSO) and various combinations of mangosteen extract and propolis extract, denoted as mangosteen extract complex (MEC) ratios, ranging from 1:0 to 0:34.<br>• The assessments included cell viability tests, evaluation of inflammatory cytokine expression via Enzyme-Linked Immunosorbent Assay (ELISA), alkaline phosphatase activity tests, alizarin red staining for mineralization, and osteoblast gene expression. | • Treatment with MEC 1:34 significantly decreased IL-6, IL-8, and PGE2 expression levels in hTERT-hNOF cells exposed to the same LPS concentration.<br>• MEC 1:34 and MEC 0:34 exhibited a higher *in vitro* bone formative effect on MG63 cells, as indicated by higher M20 levels compared to other experimental groups. Furthermore, the mangosteen extract complex induced the expression of osteoblast differentiation genes. |
| 18 | Wiwekowati et al. (2020) | • The rats were grouped into G1 (control -no orthodontic tooth movement (OTM) and no propolis), G2 (no OTM with propolis), G3 (OTM without propolis), and G4 (OTM with propolis).<br>• Five percent propolis gel was applied along with a 30gf helical spring force for OTM on rat maxilla incisors, and the treatment lasted 17 days.<br>• On day 18, blood samples were tested for MDA using ELISA. Following hematoxylin-eosin staining | • , the number of osteoblasts was calculated.<br>• The application of propolis resulted in significant differences in the number of osteoblasts compared to groups without propolis (G2>G1, G4>G3).<br>• Propolis application significantly reduced MDA serum levels compared to both groups without propolis (G2<G1; G4<G3)<br>• OTM significantly increased MDA levels compared to the control group. |
| 19 | Kresnoadi et al. (2020) | • The *Cavia cobaya* were grouped into eight, each comprising seven samples. Lower left incisors were removed and induced with polyethylene glycol (PEG), propolis extract, bovine bone graft (BBG), and a mixture of propolis extract + BBG. The animals were euthanized on days 3 and 7 postextraction.<br>• Immuno- and histological assays were conducted to detect Heat Shock Protein-70 (HSP-70) expression, osteocalcin expression, and the number of osteoblasts and osteoclasts. | • On days 3 and 7, both groups treated with the propolis extract and BBG mixture exhibited the highest levels of HSP70 and osteocalcin expression, a higher number of osteoblast cells, and a lower number of osteoclasts. |
| 20 | Handayani, Margaretha et al. (2021) | • Twenty-eight *Cavia cobaya* were divided into four groups: two control (healthy and with orthodontic tooth movement) and two treatment groups (3% and 5% propolis extract).<br>• RUNX-2 and ALP expressions were evaluated using immunohistochemical staining after 17 days. | • Increment of the RUNX-2 and ALP expression was observed on the tension side.<br>• 5% propolis extract effectively enhanced bone remodeling by increasing RUNX-2 and ALP expression during orthodontic tooth movement. |

Table 1 *(continue)*

| | References | Methodology | Results |
|---|---|---|---|
| 21 | Kresnoadi et al. (2021) | • This study used a post-test-only control group design. Fifty-six *Cavia cobaya* were randomly assigned to four groups: Control (polyethylene glycol), EEP (ethanol extract of propolis), BBG (bovine bone graft), and EEP-BBG.<br>• The left mandibular incisors were extracted, treated with the assigned materials, and sutured. BMP7 and NFATc1 expressions were observed on days 7 and 14 using immunohistochemical staining. | • On days 7 and 14, the propolis-BBG combination group had the highest BMP7 expression and the lowest NFATc1 expression.<br>• Significant differences were observed compared to the control group. BMP7 and NFATc1 expressions were strongly correlated (r = -0.598). |
| 22 | Handayani, Brahmanta et al. (2021) | • Twenty-eight male *Cavia cobaya* were divided into four groups: no orthodontic tooth movement (OTM) or propolis (K-), OTM with rubber separators (K+), OTM with 3% propolis gel (P1), and OTM with 5% propolis gel (P2).<br>• TGF-β expression in alveolar bone osteoblasts on the tension side during OTM was evaluated using immunohistochemical staining. | • TGF-β expression increased significantly with propolis gel application, with the 5% concentration being the most effective. |
| 23 | Kusumawati et al. (2021) | • Six male rabbits were periodontitis-induced using *Porphyromonas gingivalis* LPS.<br>• They were divided into three groups: Group A (Open flap debridement (OFD) only), Group B (OFD + carbonated hydroxyapatite (CHA)), and Group C (OFD + 10% propolis-CHA). CHA blocks were soaked in a 10% propolis solution for 24 hr prior to usage.<br>• On days 7 and 14, the rabbits were euthanized, and immunohistochemistry was carried out for collagen type I in tissue samples. | • Collagen type I expression was significantly higher in Group C compared to Group A and Group B on both days (p=0.000).<br>• The 10% propolis-CHA treatment increased collagen type I expression in the alveolar bone of rabbits on days 7 and 14 |
| 24 | Ari et al. (2023) | • Eighty-four *Cavia cobaya* were divided into 4 groups, each containing 7 subjects.<br>• The subjects' mandibular incisors were extracted, and the sockets were filled with either PEG (K1), propolis extract (K2), BBG (K3), or a combination of propolis extract and BBG (K4).<br>• After days 3, 7, and 30, the animals were sacrificed, and the specimens were processed to assess SMAD3 expression and measure the area of woven bone. | • All groups showed SMAD3 expression and woven bone formation.<br>• On days 3, 7, and 30, the K4 group had the highest SMAD3 expression and woven bone area. Significant differences were observed in SMAD3 expression and woven bone area across groups.<br>• The combination of propolis extract and BBG enhances bone formation by increasing SMAD3 expression and woven bone area. |

Table 1 *(continue)*

| | References | Methodology | Results |
|---|---|---|---|
| 25 | Ayyad et al. (2023) | • Eighteen male New Zealand white rabbits were used in this study. Bilateral critical-size bone defects (CSD) were created in the right and left tibias of 12 rabbits.<br>• The right tibia defects (positive control) were treated with hydroxyapatite, while the left tibia defects (study group) received a combination of hydroxyapatite (HA) and propolis. In 6 additional rabbits (negative control), CSDs were made in both tibias but left untreated.<br>• The rabbits were sacrificed at 3 and 6 weeks post-surgery for evaluation using histomorphometric analysis and light microscopy. | • At 3 weeks, the positive control group showed new bone formation, while the study group had thicker trabeculae, more organized osteocytes, and smaller marrow spaces.<br>• At 6 weeks, both groups had increased bone formation, but the study group had a higher bone surface area, though not statistically significant. The negative control group showed the least bone regeneration.<br>• Overall, HA + propolis produced better histological results than other groups. |
| 26 | Kresnoadi et al. (2023) | • Fifty-six males of *Cavia cobaya* were divided into eight groups of seven. After removing the lower left incisor, four materials — PEG, propolis extract + PEG, BBG + PEG, and propolis extract + BBG + PEG—were applied to the sockets.<br>• The animals were sacrificed at 3 and 7 days, and RANKL and osteoprotegerin (OPG) expression was examined using a light microscope at 1,000× magnification. | • On days 3 and 7, the combination group showed significantly lower RANKL and higher OPG expression.<br>• The combination of propolis extract and BBG effectively increases OPG and decreases RANKL in post-extraction sockets. |
| 27 | Colak et al. (2023) | • Twenty-nine male Wistar-Albino rats were divided into four groups: control, 35 Gy Irradiation (group 1), 35 Gy Irradiation + 100 mg/kg Propolis (group 2), and 35 Gy Irradiation + 200 mg/kg Propolis (group 3). Propolis was administered via oral gavage starting the day after radiotherapy, except for the control group.<br>• The first and second molars were extracted three weeks post-radiotherapy, and samples were collected seven weeks later.<br>• Histomorphometric analysis assessed osteoblast and osteoclast counts, and immunohistochemical analysis measured BMP-2 and TGFβ-3 levels. | • Group comparisons showed no significant differences in osteoblast and osteoclast counts.<br>• However, group 1 had the lowest and highest mean osteoblast counts.<br>• The osteoblast/osteoclast ratio differed significantly between groups, with significant differences between the control and groups 1 and 2, but not Group 3.<br>• The control group's BMP-2 and TGFβ-3 levels were significantly different from the other groups except group 3. |
| 28 | Suryono et al. (2024) | • Twenty-four samples from six male rabbits were induced with periodontitis by ligation and injected with *Porphyromonas gingivalis* LPS. | • A 10% propolis-CHA group showed a significant improvement compared to other groups. |

Table 1 *(continue)*

| | References | Methodology | Results |
|---|---|---|---|
| | | • After two weeks of periodontitis induction, the rabbits were treated and divided into three groups: group 1 (OFD only), group 2 (OFD with CHA), and group 3 (OFD with 10% propolis-CHA).<br>• Decapitation was performed on days 7 and 14 for sample collection. | |
| 29 | Fauzi et al. (2024) | • A post-test-only control group design with 30 patients is divided into two groups: one receiving *Trigona* sp. propolis after mandibular third molar extraction and the other without propolis. | • The propolis group significantly reduced pain intensity and edema from day 0 to days 1, 3, and 7.<br>• Trismus and periodontal healing improved significantly from day 0 to days 3 and 7.<br>• Trabecular values and radiographic density also showed significant improvement by week 8. |
| 30 | Askari et al. (2024) | • Electrospinning scaffolds were immersed in a propolis extract solution.<br>• Adipose-derived mesenchymal stem cells (AD-MSCs) were cultured on the scaffold and induced to differentiate into osteogenic cells.<br>• Cell viability was assessed using the MTT assay, while osteogenic differentiation was evaluated by measuring calcium levels, alkaline phosphatase (ALP) activity, and the expression of bone-specific genes. | • Cell viability was unchanged by either propolis-coated or uncoated scaffolds.<br>• However, the propolis-coated Poly (lactic-co-glycolic acid (PLGA) scaffold showed higher calcium content, ALP activity, and expression of RUNX-2, collagen type I, osteocalcin, and osteonectin on days 7, 14, and 21 of cell differentiation compared to the uncoated PLGA scaffold. |
| 31 | Florez et al. (2024) | • The 3D scaffolds were printed and impregnated with propolis extract. FTIR spectroscopy confirmed the presence of propolis.<br>• The scaffolds' mechanical strength was evaluated. Biological testing included assessing antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* using minimum inhibitory concentration (MIC), zone of inhibition (ZOI), and biofilm assays.<br>• The bmMSCs cultures were utilized to assess cell proliferation through the Alamar Blue assay, and osteogenic potential was evaluated using von Kossa, Alizarin Red, and ALP staining at various time points up to 28 days. | • Propolis impregnation maintained the scaffolds' mechanical properties, matching those of human trabecular bone.<br>• The addition of propolis provided antibacterial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis*.<br>• The presence of propolis in the scaffolds increases the cell proliferation of the bmMSCs for up to 21 days.<br>• Osteogenic potential can be seen in propolis and untreated groups. |

tropical climate there is also ideal for bee colonization, potentially involving different varieties of bees than those in the Middle East, which fosters further research. In contrast, fewer studies are conducted in North America and East Asia, likely due to challenges in obtaining propolis and a research focus that may be more centered on honey and other bee products.

### *In vivo* Studies

Among the 31 selected articles, 25 were animal studies. These investigations utilized a range of animal models, including rodents (Wistar, Sprague-Dawley, and *Cavia cobaya*), rabbits, and Mongrel dogs. The predominance of animal studies indicates a significant gap in human clinical research on propolis, suggesting the need for more human trials to confirm its efficacy and safety. The use of diverse animal models helps validate findings across species but also introduces variability, making consistent conclusions challenging. Additionally, the unspecified details in some studies point to inconsistencies in research quality and reporting standards, potentially affecting the reliability of the results.

The application of propolis in these studies was categorized into local, systemic, or a combination of both. Local applications targeted surgically-created bone defects, implants, and extracted tooth sockets, while systemic administration dosages ranged from 100 to 400 mg/kg daily, with treatment durations spanning from 3 to 56 days. Commonly assessed regions included molars, premolars, incisor areas, and long bones, such as the tibia. The primary method of evaluating outcomes in these animal studies was histological analysis, complemented by various other techniques such as immunohistochemical tests, biochemical tests, scanning electron microscopy, stereological analysis, and radiological studies.

Out of 25 animal studies, four studies evaluated the impact of propolis on the loss of alveolar bone in an in vivo periodontitis rat model (Aral et al., 2015; Kusumawati et al., 2021; Sherif et al.,2015; Suryono et al., 2024). Periodontitis is a predominant oral inflammatory disease that affects the teeth' periodontium, denoted by the formation of periodontal pockets, clinical attachment loss, and the resorption of alveolar bone. Systemic administration of propolis reduced periodontitis-related bone loss, suggesting potential benefits in periodontal treatment. (Aral et al., 2015; Sherif et al., 2015). It is supported by studies that showed an increase in the expression of TGF-β1 and collagen type I, both significant components in bone formation (Kusumawati et al., 2021; Suryono et al., 2024). However, these studies did not thoroughly investigate long-term and potential side effects.

Propolis demonstrated potential in treating alveolar bone contaminated with bacterial endotoxin, resulting in increased formation of new bone tissue (Pereira et al., 2018). Although the study indicated that propolis was applied locally, the application method was not clearly explained. This lack of detail raises concerns about the consistency and reproducibility of the

results. More recent studies show that propolis promotes bone formation by increasing the expression of key bone-related proteins, including RUNX2 and ALP (Askari, 2024; Handayani, Margaretha et al., 2021), TGF-β1 (Handayani, Brahmanta et al., 2021; Suryono, 2024), collagen type I (Kusumawati et al., 2021), and SMAD3 (Ari, 2023). It also increases osteoprotegerin (OPG) while reducing receptor activator of nuclear factor kappa-B Ligand (RANKL) (Kresnoadi, 2023) and boosts osteonectin and osteocalcin levels (Askari, 2024).

In a rat femur fracture model, systemically administered propolis was found to increase bone mineral density and enhance radiological and histological evaluation assessments, indicating its potential to accelerate fracture healing (Guney et al., 2011). Propolis was evaluated in a rabbit mandible model for its effects on bone regeneration following distraction osteogenesis. The findings indicated that propolis speeds up bone formation and could potentially reduce the consolidation phase during distraction osteogenesis (Bereket et al., 2014). Wiwekowati et al. (2020) demonstrated that propolis enhances osteogenesis during orthodontic tooth movement in rats by reducing malondialdehyde (MDA) levels and increasing the number of osteoblast cells. MDA is related to bone through its role as a marker of oxidative stress and tissue damage. High MDA levels can have implications for bone health, particularly in conditions where oxidative stress is a contributing factor. In addition, it was also shown that systemic propolis supports bone formation in a rat model of premaxillary suture expansion, which could be useful for orthodontic treatments (Altan et al., 2013). However, these results need to be interpreted with caution, as the varying quality and composition of propolis can affect the reproducibility and reliability of the findings.

In the context of dental implants, propolis-coated titanium implants in the tibiae of rabbits demonstrated earlier bone formation, mineralization, and maturation, suggesting potential benefits for implant osseointegration (Al-Molla et al., 2014). Similarly, a rat model study showed that propolis-loaded $TiO_2$ nanotubes on dental implants improved osseointegration by enhancing collagen fiber formation and reducing inflammatory cytokines (Somsanith et al., 2018). Further research in rabbits highlighted the antioxidant effects of propolis, showing improved bone healing around dental implants (Aydin et al., 2018).

When combined with bovine bone graft, propolis could preserve extraction sockets and promote bone regeneration (Kresnoadi et al., 2020). In a study involving the extraction sockets of *Cavia cobaya*, propolis combined with bovine bone graft exhibited higher expressions of heat shock protein (HSP) 70, osteocalcin, and osteoblasts, indicating improved bone regeneration (Pereira et al., 2018). The combination of propolis and NanoBone Graft in the management of dog furcation defects showed that propolis, either alone or in combination with NanoBone graft, improved bone regeneration in periodontal furcation defects (Ibrahim et al., 2019;

Zohery et al., 2017, 2018). Promising findings were also observed in a rat model of bone defects, where the combination of propolis/chitosan/decalcified bone matrix scaffolds led to significant improvements in bone formation (Meimandi-Parizi et al., 2018).

While the result from animal studies suggests that propolis has significant potential in enhancing bone regeneration and implant osseointegration, several limitations must be considered, as the research has been conducted on animal models, which may not fully replicate the complexities of human physiology and bone healing processes. This difference can impact the generalizability of the findings to human clinical scenarios. Furthermore, propolis's long-term effects and safety in humans are not fully understood. Therefore, further research, including well-designed clinical trials, is essential to confirm these effects in humans and establish safe and effective guidelines for using propolis in dental and orthopedic applications. Based on our keyword-specific search, only one study used human samples to assess the effects of propolis on extraction sockets (Fauzi et al., 2024). The results indicated that the pain and edema significantly improved within 7 days, and trabecular values and radiographic density significantly improved by 8 weeks.

### *In vitro* Studies

Only five of the selected articles presented are *in vitro* studies. These studies employed different cell types, including human gingival fibroblasts (hTERT-hNOF) cell line, human peripheral blood mononuclear cells, osteoclast-like cells derived from the murine cell line, adipose-derived mesenchymal stem cells and bone marrow mesenchymal stem cells. In these studies, propolis was either administered as a supplement within the cell culture media or coated on a titanium implant or scaffold. The in vitro results indicated that propolis supplementation may stimulate bone formation (Askari et al., 2024; Florez et al., 2024; Lim et al., 2019) and inhibit osteoclastogenesis (Pillegi et al., 2018; Wimolsantirungsri et al., 2018). Moreover, propolis was found to reduce the expression of inflammatory cytokines, which then promote in vitro bone formation in human gingival fibroblasts (Lim et al., 2019). Another study using human peripheral blood mononuclear cells showed propolis reduced osteoclast formation, suggesting anti-osteoclastogenic effects (Pillegi et al., 2018).

The outcomes of the in vitro studies strongly corroborated the *in vivo* findings, demonstrating a reduction in inflammation, an increase in bone formation, and a significant anti-osteoclastogenic effect. However, it is important to note that in vitro studies, while valuable, have limitations in replicating the complex biological environment of a living organism. The cell types and culture conditions may not fully represent the *in vivo* situation. Additionally, the dosage used in vitro cannot be translated directly to the dosage used *in vivo* without considering the pharmacokinetics aspects of propolis, which can affect the applicability of the results.

## CONCLUSION

This review encompasses animal studies and in vitro research, highlighting the potential advantages of propolis in promoting bone health and facilitating the healing process. Remarkably, both systemic and local administrations of propolis have been linked to decreased alveolar bone loss, heightened bone mineral content and density, enhanced osseointegration of dental implants, and expedited bone healing across diverse experimental models. Moreover, propolis exhibits anti-inflammatory and antioxidant attributes, amplifying its therapeutic efficacy in addressing inflammatory bone disorders like periodontitis. Incorporating propolis in bone healing strategies offers a cost-efficient approach utilizing its natural, anti-inflammatory, antimicrobial, and bone-regenerative properties. It will reduce the need for expensive synthetic drugs and extended treatments and enhance patient outcomes and healthcare efficiency.

These findings support the potential of propolis as a natural therapeutic agent with promising applications in dentistry and medicine. Its multifaceted impact on bone health, including the modulation of various stages of the bone healing process, warrants significant consideration for future research and clinical implementation. However, further investigations, particularly well-designed randomized clinical trials, are essential to confirm the efficacy and safety of propolis in human applications. In conclusion, this review highlights that propolis may become a valuable contributor to improving bone healing and managing bone-related conditions in the future. Nonetheless, the current review may be limited by the selection of keywords used to search for relevant studies, which could have affected the comprehensiveness of the research covered.

## ACKNOWLEDGEMENT

This work was supported by the School of Dental Sciences, Universiti Sains Malaysia, Kubang Kerian Kelantan, Malaysia.

## REFERENCES

Al-Molla, B. H., Al-Ghaban, N., & Taher, A. (2014). Immunohistochemical evaluation: The effects of propolis on osseointegration of dental implants in rabbit's tibia. *Journal of Dental Research and Review*, *1*(3), 123-131. https://doi.org/10.4103/2348-2915.146490

Almeida, E. C. D., & Menezes, H. (2002). Anti-inflammatory activity of propolis extracts: A review. *Journal of Venomous Animals and Toxins*, *8*(2), 191-212. https://doi.org/10.1590/S0104-79302002000200002

Altan, B. A., Kara, I. M., Nalcaci, R., Ozan, F., Erdogan, S. M., Ozkut, M. M., & Inan, S. (2013). Systemic propolis stimulates new bone formation at the expanded suture: A histomorphometric study. *Angle Orthodontist*, *83*(2), 286–291. https://doi.org/10.2319/032612-253.1

Aral, C. A., Kesim, S., Greenwell, H., Kara, M., Çetin, A., & Yakan, B. (2015). Alveolar bone protective and hypoglycemic effects of systemic propolis treatment in experimental periodontitis and diabetes mellitus. *Journal of Medicinal Food*, *18*(2), 195–201. https://doi.org/10.1089/jmf.2013.3137

Ari, M. D. A., Kresnoadi, U., Azhar, I. S., Sari, N., & Riawan, W. (2023). Alveolar bone formation enhancement induced by the combination of

propolis extract and bovine bone graft through SMAD3 expression and woven bone in the socket preservation (an *in vivo* study). *Research Journal of Pharmacy and Technology*, *16*(10), 4805-4810. https://doi.org/10.52711/0974-360X.2023.00779

Askari, M., Jadid Tavaf, M., Ghorbani, M., Yazdanian, M., & Moghaddam, M. M. (2024). Electrospun propolis-coated PLGA scaffold enhances the osteoinduction of mesenchymal stem cells. *Current Stem Cell Research and Therapy*, *19*(1), 94-102. https://doi.org/10.2174/1574888X18666230330085615

Aydin, E., Hepokur, C., Misir, S., & Yeler, H. (2018). Effects of propolis on oxidative stress in rabbits undergoing implant surgery. *Cumhuriyet Dental Journal*, *21*(2), 136–144. https://doi.org/10.7126/cumudj.356554

Ayyad, A., Mohamed, N., Melek, L., & Khalil, N. (2023). Effects of the combination of propolis and hydroxyapatite on bone regeneration (an experimental animal study). *Alexandria Dental Journal*, *48*(2), 95-103. https://doi.org/10.21608/adjalexu.2022.140038.1281

Bereket, C., Özan, F., Şener, I., Tek, M., Altunkaynak, B., Z., Semirgin, S., U., Senel, E., & Ozdemir, M. (2014). Propolis accelerates the consolidation phase in distraction osteogenesis. *Journal of Craniofacial Surgery*, *25*(5), 1912–1916. https://doi.org/10.1097/SCS.0000000000000946

Çolak, S., Erdil, A., & Gevrek, F. (2023). Effects of systemic Anatolian propolis administration on a rat-irradiated osteoradionecrosis model. *Journal of Applied Oral Science*, *31*, e20230231. https://doi.org/10.1590/1678-7757-2023-0231

El-Haskoury, R., Al-Waili, N., Kamoun, Z., Makni, M., Al-Waili, A., & Lyoussi, B. (2021). Antioxidant activity and protective effect of propolis against carbon tetrachloride-induced liver and kidney injury by modulation of oxidative parameters. *Veterinary World*, *14*(12), 3076–3083. https://doi.org/10.14202/vetworld.2021.3076-3083

Fauzi, A., Rasul, I., Chandha M. H., Sabir, A., Yudha, G.A.W., (2024) *Effectiveness of propolis application in wound healing of third molar mandibula post odontectomy (clinical and radiographic analysis) - A comparative study*. Research Square. https://doi.org/10.21203/rs.3.rs-4392913/v1

Florez, A. I. M., Malagon, S., Ocampo, S., Leal-Marin, S., Ossa, E. A., Glasmacher, B., Garcia, C., & Pelaez-Vargas, A. (2024). *In vitro* evaluation of the osteogenic and antimicrobial potential of porous wollastonite scaffolds impregnated with ethanolic extracts of propolis. *Frontiers in Bioengineering and Biotechnology*, *12*, 1321466. https://doi.org/10.3389/fbioe.2024.1321466

Guney, A., Karaman, I., Oner, M., & Yerer, M. B. (2011) Effects of propolis on fracture healing: An experimental study. *Phytotherapy Research*, *25*(11), 1648-1652. https://doi.org/10.1002/ptr.3470

Handayani, B., Margaretha, M. S. A., Ridwan, R. D., & Aljunaid, M. (2021). The effect of apis mellifera propolis on RUNX-2 and ALP during remodeling of orthodontic tooth movement. *Research Journal of Pharmacy and Technology*, *14*(5), 2363-2366. https://doi.org/10.52711/0974-360X.2021.00417

Handayani, B., Brahmanta, A., Prameswari, N., & Syahdinda, M. R. (2021). Transforming growth factor-β expression in the tension side of orthodontic tooth movement after local administration of propolis gel extract. *Biochemical and Cellular Archives*, *21*(1), 1859-1862.

Ibrahim, M., Khalil, N. M, & Fahmy, R. A. (2019). Management of class II furcation defects in dogs using combination of propolis and nanobone graft. *Egyptian Dental Journal*, *65*(4), 3365–3374. https://doi.org/10.21608/EDJ.2019.74774

Kresnoadi, U., Rahayu, R. P., Ariani, M. D., & Soesanto, S. (2020). The potential of natural propolis extract combined with bovine bone graft in increasing heat shock protein 70 and osteocalcin on socket preservation. *European Journal of Dentistry*, *14*(1), 031–037. https://doi.org/10.1055/s-0040-1701921

Kresnoadi, U., Sari, N., & Laksono, H. (2023). Socket preservation using a combination of propolis extract and bovine bone graft towards the expression of receptor activator of nuclear κB ligand and osteoprogerin. *Folia Medica*, *65*(5), 737-743. https://doi.org/10.3897/folmed.65.e95802

Kresnoadi, U., Widjaja, J., & Laksono, H. (2021). Ethanol extract of propolis-bovine bone graft combination as a prospective candidate for socket preservation: Enhancing BMP7 and decreasing NFATc1. *The Saudi Dental Journal*, *33*(8), 1055-1062. https://doi.org/10.1016/j.sdentj.2021.05.003

Kuropatnicki, A. K., Szliszka E., & Krol, W. (2013). Historical aspects of propolis research in modern times. *Evidence-Based Complementary and Alternative Medicine*, *2013*(1), 964149. https://doi.org/10.1155/2013/964149.

Kusumawati, I., Suryono, S., & Syaify, A. (2021). The enhancement of collagen type I expression after 10% propolis-carbonated hydroxyapatite application in periodontitis-induced rabbits. *Dental Journal*, *54*(1), 16–20. https://doi.org/10.20473/j.djmkg.v54.i1.p16-20

Lim, Y. K., Yoo, S. Y., Jang, Y. Y,, Lee, B. C., Lee, D. S., & Kook, J. K. (2019). Anti-inflammatory and in vitro bone formation effects of *Garcinia mangostana* L. and propolis extracts. *Food Science and Biotechnology*, *29*(4), 539–48. https://doi.org/10.1007/s10068-019-00697-3

Lotfy, M. (2006). Biological activity of bee propolis in health and disease. *Asian Pacific Journal of Cancer Prevention*, *7*(1), 22–31.

Marcucci, M. C. (1995). Propolis: Chemical composition, biological properties and therapeutic activity. *Apidologie*, *26*(2), 83–99. https://doi.org/10.1051/apido:19950202

Meimandi-Parizi, A., Oryan, A., Sayahi, E., & Bigham-Sadegh, A. (2018). Propolis extract a new reinforcement material in improving bone healing: An *in vivo* study. *International Journal of Surgery*, *56*, 94–101. https://doi.org/10.1016/j.ijsu.2018.06.006

Oršolić, N., & Jembrek, M. J. (2022). Molecular and cellular mechanisms of propolis and its polyphenolic compounds against cancer. *International Journal of Molecular Sciences*, *23*(18), 10479. https://doi: 10.3390/ijms231810479

Pereira, Y. C. L., Issa, J. P. M., Watanabe, E., Nascimento, G. C., Iyomasa, M. M., Del Ciampo, J. O., & Ervolino E. (2018). The therapeutic use of propolis extract in alveolar bone contaminated with bacterial endotoxin. *Dentistry*, *8*(473), 2161-1122. https://doi.org/10.4172/2161-1122.1000473

Pileggi, R., Antony, K., Johnson, K., Zuo, J., & Holliday, L. S. (2009). Propolis inhibits osteoclast maturation. *Dental Traumatology*, *25*(6), 584–588. https://doi.org/10.1111/j.1600-9657.2009.00821.x

Sherif, H., El Sawa, A., & Karam, S. (2015). Effect of propolis on induced alveolar bone loss in diabetic rats. *Alexandria Dental Journal*, *40*(2), 242–248. https://doi.org/0.21608/adjalexu.2015.59160

Somsanith, N., Kim, Y. K., Jang, Y. S., Lee, Y. H., Yi, H. K., Jang, J. H., Kim, K. A., Bae, T. S., & Lee, M. H. (2018). Enhancing of osseointegration with propolis-loaded $TiO_2$ nanotubes in rat mandible for dental implants. *Materials*, *11*(1), 61. https://doi.org/10.3390/ma11010061

Steppe, L., Megafu, M., Tschaffon-Müller, M. E. A., Ignatius, A., & Haffner-Luntzer, M. (2023). Fracture healing research: Recent insights. *Bone*

*Reports*, *19*, 101686. https://doi.org/10.1016/j.bonr.2023.101686

Suryono, Wijayanti, P., & Lastianny, S. P. (2024). The effect of carbonated hidroxyapatite-10% propolis application on open flap debridement towards transforming growth factor β1 expression. *Malaysian Journal of Medicine and Health Sciences*, *20*(2), 45-51

Toker, H., Ozan, F., Ozer, H., Ozdemir, H., Eren, K., & Yeler, H. (2008). A morphometric and histopathologic evaluation of the effects of propolis on alveolar bone loss in experimental periodontitis in rats. *Journal of Periodontology*, *79*(6), 1089–1094. https://doi.org/10.1902/jop.2008.070462

Tolay, M. M., Sacchetto, J., Massad, W. A., Airaghi, F. L., González, M., & Natera, J. E., (2024). Yungas propolis in the scavenging of vitamin B2-photogenerated ROS. *Journal of Photochemistry and Photobiology A: Chemistry, 452*, 115611. https://doi.org/10.1016/j.jphotochem.2024.115611

Wimolsantirungsri, N., Makeudom, A., Louwakul, P., Sastraruji, T., Chailertvanitkul, P., Supanchart, C., & Krisanaprakornkit, S. (2018). Inhibitory effect of Thai propolis on human osteoclastogenesis. *Dental Traumatology*, *34*(4), 237–244. https://doi.org/10.1111/edt.12401

Wiwekowati, W., Ma'ruf, M. T., Walianto, S., Sabir, A., & Widyadharma, I. P. E. (2020). Indonesian propolis reduces malondialdehyde level and increase osteoblast cell number in alveolar bone of wistar rats with orthodontic tooth movement. *Open Access Macedonian Journal of Medical Sciences*, *8*(A), 100-104. https://doi.org/10.3889/oamjms

Zabaiou, N., Fouache, A., Trousson, A., Baron, S., Zellagui, A., Lahouel, M., & Lobaccaro, J. A. (2017). Biological properties of propolis extracts: Something new from an ancient product. *Chemistry and Physics of Lipids*, *207*(Part B), 214–222. https://doi.org/10.1016/j.chemphyslip.2017.04.005

Zohery, A. A., Meshri, S. M., Madi, M. I., Rehim, S. S. A. E., & Nour, Z. M. (2018). Egyptian propolis compared to nanohydroxyapatite graft in the treatment of class II furcation defects in dogs. *Journal of Periodontology*, *89*(11), 1340–1350. https://doi.org/10.1002/JPER.17-0685

Zohery, A. A., Nour, Z. M., Rehim, S. S. A. E., & Mady, M. I. (2017). Histomorphometric analysis of bone regeneration after use of propolis versus nanobone graft materials for the management of class II furcation defects in dogs. *Alexandria Dental Journal*, *42*(2), 198–203. https://doi.org/10.21608/adjalexu.2017.57927

Zulhendri, F., Lesmana, R., Tandean, S., Christoper, A., Chandrasekaran, K., Irsyam, I., Suwantika, A. A., Abdulah, R., & Wathoni, N. (2022). Recent update on the anti-inflammatory activities of propolis. *Molecules*, *27*(23), 8473. https://doi.org/10.3390/molecules27238473

# REFEREES FOR THE PERTANIKA
## JOURNAL OF TROPICAL AGRICULTURAL SCIENCE

### Vol. 47 (4) Nov. 2024

The Editorial Board of the Pertanika Journal of Tropical Agricultural Science wishes to thank the following:

Abdul Sattar
*(BZU, Pakistan)*

Adibah Mohd Amin
*(UPM, Malaysia)*

Ahmad N. Al-Baarri
*(UNDIP, Indonesia)*

Alimuddin Ali
*(UNM, Indonesia)*

Amilia Aminuddin
*(UKM, Malaysia)*

Basripuzi Nurul Hayyan Hassan Basri
*(UMK, Malaysia)*

Ch'ng Huck Ywih
*(UMK, Malaysia)*

Diny Agustini Sandrasari
*(IPB, Indonesia)*

Ernestina Valadez-Moctezuma
*(UACh, Mexico)*

Fazal Ullah
*(LZU, China)*

Farida Habib Khan
*(University of Ha'il, Kingdom of Saudi Arabia)*

Giuseppe Capobianco
*(Sapienza, Italy)*

Hseu Zeng Yei
*(NTU, Taiwan)*

Indika Herath
*(WUSL, Sri Lanka)*

Indra Purnama
*(Unilak, Indonesia)*

Izzati Adilah Azmir
*(UiTM, Malaysia)*

Jamsari
*(Unand, Indonesia)*

Janice Alano Ragaza
*(Ateneo, Phillipnes)*

Javad Seyedmohammadi
*(AREEO, Iran)*

Jelena Vranesevic
*(NIVNS, Serbia)*

Joham Sarfaraz Ali
*(NUMS, Pakistan)*

Jung Chuleui
*(ANU, Korea)*

Khanitta Somtrakoon
*(MSU, Thailand)*

Khor Kuan Hua
*(UPM, Malaysia)*

Kok Yong Chin
*(UKM, Malaysia)*

Kyung-Min Kim
*(KNU, Korea)*

Lam Ngoc Thi Thai
*(VinhUni, Vietnam)*

Lubabalo Bilaa
*(UL, Africa)*

Lim Leong-Seng
*(UMS, Malaysia)*

Lum Mok Sam
*(UMS, Malaysia)*

Lusia Komala Widiastuti
*(Unila, Indonesia)*

Mai Thanh Thi Nguyen
*(VinhUni, Vietnam)*

Mami Irie
*(Nodai, Japan)*

Martini Mohammad Yusoff
*(UPM, Malaysia)*

Mohammad Noor Azmai Amal
*(UPM, Malaysia)*

Mohd Nizam Lani
*(UMT, Malaysia)*

Monton Visutthi
*(NRRU, Thailand)*

Muhamad Hazim Nazli
*(UPM, Malaysia)*

Muhammad Firdaus Sulaiman
*(UPM, Malaysia)*

Munirah Yaacob
*(IIUM, Malaysia)*

Najiah Musa
*(UMT, Malaysia)*

Nazia Abdul Kadar
*(UMS, Malaysia)*

Neni Kartini Che Mohd Ramli
*(UiTM, Malaysia)*

Nguyen Huu Hien
*(VinhUni, Vietnam)*

Noppawan Nounjan
*(KKU, Thailand)*

Nor Azizun Rusdi
*(UMS, Malaysia)*

Nor Elliza Tajidin
*(UPM, Malaysia)*

Norhayati Yusuf
*(UMT, Malaysia)*

Noureddine Benkeblia
*(UWI, Jamaica)*

Nur Hafizah Azizan
*(IIUM, Malaysia)*

Paquito Enclonar Relox
*(DNSC, Phillipines)*

Phebe Ding
*(UPM, Malaysia)*

Rina Arimarsetiowati
*(Puslitkoka, Indonesia)*

Rosimah Nulit
*(UPM, Malaysia)*

Ruhil Hayati Hamdan
*(UMK, Malaysia)*

Sharifah Raina Manaf
*(UiTM, Malaysia)*

Sharifah Syed Hassan
*(Monash, Malaysia)*

Sharina Omar
*(UPM, Malaysia)*

Siti Noor Hajjar Md Latip
*(UiTM, Malaysia)*

Surisa Phornvillay
*(UNIMAS, Malaysia)*

Tan Geok Hun
*(UPM, Malaysia)*

Tati Suryati Syamsudin
*(ITB, Indonesia)*

Wafaa Abd El-Ghany
*(CU, Egypt)*

Wan Asrina Wan Yahaya
*(UPM, Malaysia)*

Weeradej Meeinkuirt
*(MU, Thailand)*

Wirdhatul Muslihatin
*(ITS, Indonesia)*

Yavar Vafaee
*(UKH, Iraq)*

| | |
|---|---|
| *ANU* | *– Andong National University* |
| *AREEO* | *– Agricultural Research, Education and Extension Organization* |
| *Ateneo* | *– Ateneo de Manila University* |
| *BRIN* | *– Badan Riset dan Inovasi Nasional* |
| *BZU* | *– Bahauddin Zakariya University* |
| *CU* | *– Cairo University* |
| *DNSC* | *– Davao del Norte State College* |
| *KKU* | *– Khon Kaen University* |
| *KNU* | *– Kyungpook National University* |
| *IIUM* | *– International Islamic University Malaysia* |
| *IPB* | *– Institut Pertanian Bogor* |
| *ITB* | *– Institut Teknologi Bandung* |
| *ITS* | *– Institute Technology Sepuluh Nopember* |
| *LZU* | *– Lanzhou University* |
| *Monash* | *– Monash University Malaysia* |
| *MSU* | *– Mahasarakham University* |
| *MU* | *– Mahidol University* |
| *NIVNS* | *– Novi Sad Naučni Institut Za Veterinarstvo* |
| *Nodai* | *– Tokyo University of Agriculture* |
| *NRRU* | *– Nakhon Ratchasima Rajabhat University* |
| *NTU* | *– National Taiwan University* |
| *NUMS* | *– National University of Medical Sciences* |
| *Puslitkoka* | *– Pusat Penelitian Kopi dan Kakao* |
| *Sapienza* | *– Sapienza – Università di Roma* |
| *UACh* | *– Universidad Autónoma Chapingo* |
| *UiTM* | *– Universiti Teknologi MARA* |
| *UKH* | *– University of Kurdistan Hewlêr* |
| *UKM* | *- Universiti Kebangsaan Malaysia* |
| *UL* | *– Universiteit van Limpopo* |
| *UMK* | *– Universiti Malaysia Kelantan* |
| *UMS* | *– Universiti Malaysia Sabah* |
| *UMT* | *– Universiti Malaysia Terengganu* |
| *Unand* | *– Universitas Andalas* |
| *UNDIP* | *– Universitas Diponegoro* |
| *Unila* | *– Universitas Lampung* |
| *Unilak* | *– Universitas Lancang Kuning* |
| *UNM* | *– Universitas Negeri Makassar* |
| *UNM* | *– University of Nottingham* |
| *UPM* | *– Universiti Putra Malaysia* |
| *UWI* | *– University of the West Indies* |
| *VinhUni* | *– Vinh University* |
| *WUSL* | *– Wayamba University of Sri Lanka* |

While every effort has been made to include a complete list of referees for the period stated above, however if any name(s) have been omitted unintentionally or spelt incorrectly, please notify the Chief Executive Editor, *Pertanika* Journals at executive_editor.pertanika@upm.edu.my

Any inclusion or exclusion of name(s) on this page does not commit the *Pertanika* Editorial Office, nor the UPM Press or the university to provide any liability for whatsoever reason.

**Selected papers from the 11th International Conference on Multidisciplinary Research (2023)**
Guest Editor: Enis Nadia Md Yusof

Pertanika Journal of Tropical Agricultural Science
Vol. 47 (4) Nov. 2024

## Content



**Pertanika Editorial Office, Journal Division,**
Putra Science Park,
1st Floor, IDEA Tower II,
UPM-MTDC Center,
Universiti Putra Malaysia,
43400 UPM Serdang,
Selangor Darul Ehsan
Malaysia

http://www.pertanika.upm.edu.my
Email: executive_editor.pertanika@upm.edu.my
Tel. No.: +603- 9769 1622

PENERBIT UPM PRESS

http://www.penerbit.upm.edu.my
Email: penerbit@upm.edu.my
Tel. No.: +603- 9769 8851

e-ISSN 2231-8542
ISSN 1511-3701