Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

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PERTANIKA JOURNALS

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Journal of Tropical Agricultural Science

About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science (JTAS) is the official journal of Universiti Putra Malaysia published by UPM Press. It is an open-access online scientific journal which is free of charge. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognized 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.

JTAS 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 to authors around the world regardless of the nationality.

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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.

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Pertanika was founded in 1978. A decision was made in 1992 to streamline Pertanika into three journals as Journal of Tropical Agricultural Science, Journal of Science & Technology, and Journal of Social Sciences & Humanities to meet the need for specialised journals in areas of study aligned with the interdisciplinary strengths of the university.

After 40 years, as an interdisciplinary journal of Agriculture, the revamped Journal, a leading agricultural journal in Malaysia now focuses on tropical agricultural research and its related fields.

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The *Introduction* explains the scope and objective of the study in the light of current knowledge on the subject; the *Materials and Methods* describes how the study was conducted; the *Results* section reports what was found in the study; and the *Discussion* section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the Journal's **Instructions to Authors**.

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Notification of the editorial decision is usually provided within ten to fourteen weeks 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.

As articles are double-blind reviewed, material that might identify authorship of the paper should be placed only on page 2 as described in the first-4 page format in Pertanika's **Instructions to Authors** given at the back of this journal.

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

1. The Journal's chief executive editor and the editorial board examine the paper to determine whether it is appropriate for the journal and should be reviewed. 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 three reviewers. Typically, one of these is from the Journal's editorial board. Others are specialists in the subject matter represented by the article. The chief executive editor asks them to complete the review in three weeks.

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3. The chief executive editor, in consultation with the editor-in-chief, examines the reviews and decides whether to reject the manuscript, invite the author(s) to revise and resubmit the manuscript, or seek additional reviews. Final acceptance or rejection rests with the Edito-in-Chief, who reserves the right to refuse any material for publication. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the author) are forwarded to the author. If a revision is indicated, the editor provides guidelines 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 author(s) may also submit a rebuttal if there is a need especially when the author disagrees with certain comments provided by reviewer(s).

5. The chief executive editor sends the revised paper out for re-review. Typically, at least one of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the chief executive editor in consultation with the editorial board and the editor-in-chief examine their comments and decide whether the paper is ready to be published, needs another round of revisions, or should be rejected.

7. If the decision is to accept, an acceptance letter is sent to all the author(s), the paper is sent to the Press. The article should appear in print in approximately three months.

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Foreword

Welcome to the First Issue of 2019 for the Journal of Tropical Agricultural Science (JTAS)!

JTAS 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 and run on a non-profit basis for the benefit of the world-wide science community.

This issue contains 28 articles; 3 are review articles, 1 is a short communication and the rest are regular articles. The authors of these articles come from different countries namely Bangladesh, Indonesia, Iran, Iraq, Malaysia, Nigeria, Philippines and Thailand.

Articles submitted in this issue cover various scopes of Tropical Agricultural Science including animal production, aquaculture, biochemistry, biotechnology, crop and pasture production, ecology, fisheries sciences, food and nutrition development, forestry sciences, genetics and molecular biology, horticulture, microbiology, plant physiology, soil and water sciences and zoology.

Selected from the scope of food and nutrition development is an article entitled "Antioxidant and Antidiabetic Effects of *Garcinia schomburgkiana* Extracts and Fermented Juices" by Sirikul Thummajitsakul, Bongkoj Boonburapong and Kun Silprasit, fellow researchers from Srinakharinwirot University, Thailand. Their study investigated total phenolic contents, antioxidant activities, and antidiabetic activities in flesh, seed and leaf extracts, and the fermented juices of *Garcinia schomburgkiana* Pierre (Madan). They found that the seed extract showed the highest value of total phenolic content, radical scavenging activity, and amylase inhibitory activity, whereas the leaf extracts revealed the strongest ferrous ion chelating activity, and inhibitory activities on glucosidase and lipase. Their results also indicated that *G. schomburgkiana* Pierre is a potential nutraceutical source, and its fermented juice can be further improved as healthy fruit drink. Details of the article is available on page 45.

Selected from the scope of biotechnology is an article entitled "Removal of Heavy Metals in Lake Water Using Bioflocculant Produced by *Bacillus subtilis"* by Choong Chiou Dih, Nurul Amirah Jamaluddin and Zufarzaana Zulkeflee, fellow researchers from Faculty of Environmental Studies, Universiti Putra Malaysia, Malaysia. The study investigated the removal of heavy metals from synthetic wastewater and lake water samples that had been treated with bioflocculant produced by the strain. ICP-MS was used to measure the concentration of the metals in the samples before and after treatment by the

bioflocculant. Their results had proven that bioflocculant produced by *B. subtilis* was a good alternative to chemically-based solution in remediating heavy metal polluted waters. Details of the article is available on page 89.

Selected from the scope of microbiology is an article entitled "Biorisk Assessment of Antibiotic-Resistant Pathogenic Bacteria Isolated from Swiftlet Houses in Sarawak" by Sui Sien Leong, Lihan Samuel, Hwa Chuan Chia, Jui Heng Roland Kueh and Yee Min Kwan**,** fellow researchers from Universiti Malaysia Sarawak, Malaysia. The study investigated the relative effects of the main determinants of antibiotic-resistant pathogenic bacteria and estimated the risk of the emergence and spread of antibiotic resistance among humans in the swiftlet's faeces and its indoor air to human health. The methods applied focused mainly on the hazard identification, exposure assessment, and hazard assessment. Their results showed that over half of the pathogenic bacterial isolates were multidrug-resistant to a wide range of commonly used antibiotics. These emerging pathogenic antibiotic resistant bacteria are capable of causing life-threatening infections which pose a health hazard to the biodiversity. Details of the article is available on page 285.

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 and editors, who have made this issue possible.

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

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Review Article

Overview of the Pharmacological Activities of *Aframomum melegueta*

Edwin Yu Sheng Toh, Chooi Ling Lim, Anna Pick Kiong Ling, Soi Moi Chye and Rhun Yian Koh*

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ABSTRACT

Aframomum melegueta (AM) is an herbaceous plant consumed as an edible spice and traditionally used to treat common ailments in West Africa, such as body pains, diarrhoea, sore throat, catarrh, congestion and rheumatism. Moreover, AM has been used to treat infectious diseases such as urinary tract infections caused by *Escherichia coli, Klebsiella pneumoniae, Enterococcus faecalis, Staphylococcus saprophyticus, Proteus mirabilis,* methicillin-resistant *Staphylococcus aureus*, *Salmonella* spp, and *Shigella* spp. Based on current literature, different parts of the plant possess specific phytochemicals such as flavonoids, phenolic compounds, alkaloids, tannins, terpenoids, saponins, and cardiac glycosides that have healing potential and medicinal purposes. These phytochemicals exhibit anti-inflammatory, antimicrobial, anti-allergic, anti-clotting, anti-cancer, anti-diabetic and hepatoprotective effects. They also act as antioxidants to counteract free radicals, and immune enhancers as well as hormone modulators. However, research on medicinal properties of AM is still very limited. Therefore, more comprehensive studies need to be performed to elucidate the medicinal purposes of AM. This review summarises findings from previous studies on the pharmacological activities of AM.

Keywords: Aframomum melegueta, natural product, pharmacological activity, phytochemicals

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INTRODUCTION

Tropical plants have, since antiquity, been known to exhibit pharmacological effects, and utilising them for therapeutic purposes is far more economical than allopathic medicine. A report has suggested that the total number of terrestrial plant species is around 500,000 species globally (Corlett, 2016), consisting of an estimated 450,000 angiosperms (Pimm & Joppa, 2015), 1,000 gymnosperms (Christenhusz et al., 2011), 10,000 ferns (Ranker & Sundue, 2015), 1,300 lycophytes, 9,000 mosses (Magill, 2010), 250 hornworts (Villarreal et al., 2010) and 7,500 liverworts (Von Konrat et al., 2010). The practice of using plants to treat diseases has originated since antiquity, when people discovered that they harbour healing potentials (Ríos & Recio, 2005). The practice of using plants for the maintenance of health or treatment of illness is regarded as a type of "traditional medicine". The term "traditional medicine" is interchangeably used with "herbal medicine" or "natural medicine" (Gilani & Rahman, 2005).

In the same light, *Aframomum melegueta* (AM), from the family Zingiberaceae, is a shining example of an herbaceous plant used traditionally for treating ailments. The taxonomical classification of the plant is as follows: Plantae (kingdom), Tracheophyta (phylum), Liliopsida (class), Zingiberales (order), Zingiberaceae (family), *Aframomum* (genus) and *Aframomum melegueta* (species). This plant is also known as alligator pepper, grains of paradise, guinea pepper or melegueta pepper. It is native to tropical African countries such as Ghana, Nigeria, Liberia and Cameroon (Ngwoke et al., 2014), and is an important commercial crop in east African countries such as Ethiopia. It is cultivated in Indian house gardens as well (Khare, 2007). This plant can grow up to 1.5 m in height with orange-coloured lips and pinkish-orange upper flowers that can develop into fleshly and indehiscent pods. The size of the pods are 5-7 cm in length, are edible and contain numerous small, reddish brown seeds (Figure 1) with a pungent scent of ginger and cayenne pepper. The stem is short and covered with scars of fallen leaves. The leaves are about 30 cm long, 12 cm wide, and have close nerves underneath (Ilic et al., 2010; Van Harten, 1970).

A common condiment in West and North African cuisine, melegueta pepper or AM has been used as a spice for meats, sauces and soups. Traditionally, AM is mixed with other herbs for the treatment of common ailments such as body pains, diarrhoea, sore throat, catarrh, congestion and rheumatism in West Africa (Ajaiyeoba & Ekundayo, 1999). It is a perennial (seasonless) herbal plant that is often cultivated owing to its valuable pharmacological effects such as antimicrobial, hepato-protective, anti-cancer and anti-diabetic effects (Bravo, 1998; El-Halawany et al., 2014; Mohammed et al., 2017; Ngwoke et al., 2014). *In lieu* of mounting interest in the plant's bioactive effects, this review summarises some of the major pharmacological activities of AM.

Figure 1. Seeds of *Aframomum melegueta*

PHYTOCHEMICALS IN *Aframomum melegueta*

As with essentially all medicinal plants, the therapeutic effects of AM are generally due to the presence of secondary metabolites, known as phytochemicals (Alphonso & Saraf, 2012). Secondary metabolites are produced in response to stress factors and may not be involved in the normal growth and development of the plants. For example, hydroxylated coumarins are accumulated in carrots in response to fungal invasion (Darvill & Albersheim, 1984). Flavonoids are the most abundant compounds present in AM, which act as powerful antioxidants to alleviate medical conditions. Other compounds include phenols, saponins, tannins, ascorbic acid, niacin, riboflavin, thiamine, and minerals such as calcium, phosphorus, potassium, magnesium, sodium, iron, zinc, manganese and copper (Okwu, 2004, 2005). Table 1 shows the common phytochemicals present in AM and their common medicinal purposes. Active compounds that are identified in AM include gingerol, shogaol, paradol, rac-6-dihydroparadol and gingeredione, which demonstrate antimicrobial potential (Odetunde et al., 2015). Chemical structures of the active compounds are shown in Figure 2.

Table 1

Common phytochemicals in Aframomum melegueta (Okwu, 2004, 2005) and their potential medicinal properties

Phytochemicals	Potential medicinal properties
Flavonoids	Treat allergies (Castell et al., 2014), inflammation and intestinal troubles (Salaritabar et al., 2017) Counter free radicals, microbes (Cushnie & Lamb, 2005), ulcers (Mota et al., 2009), viruses, hepatoxins (Davila et al., 1989) Cause platelet aggregation (Faggio et al., 2017) Exhibit anti-cancer properties (Batra & Sharma, 2013)
Phenolic compounds	Promote wound healing (Song et al., 2017) and prevent infections (Machado et al., 2018) Exhibit anti-clotting, anti-inflammatory and antioxidant effects (Olas et al., 2008) Act as immune enhancer (Cuevas et al., 2013) as well as hormone modulator (Venugopal & Liu, 2012)
Alkaloids	Act as analgesic (Sutradhar et al., 2007), antispasmodic (Calixto et al., 1984) and bactericidal (Cushnie et al., 2014) agents

Table 1 *(Continue)*

Phytochemicals	Potential medicinal properties
Tannins	Hasten the healing of wounds and inhibit inflamed mucous membrane (Su et al., 2017)

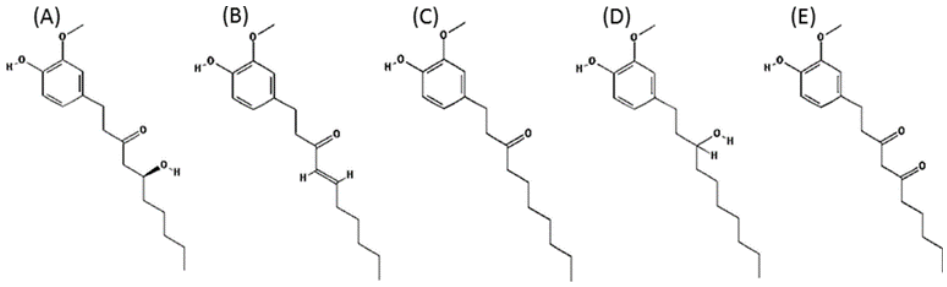

Figure 2. Chemical structures of (A) Gingerol, (B) Shogaol, (C) Paradol, (D) Rac-6-Dihydroparadol and (E) Gingeredione

Medicinal Properties of *Aframomum melegueta*

Antimicrobial Effect

One of the most significant pharmacological effects of AM is its antimicrobial activity. AM has been used to treat urinary tract as well as soft tissue infections, thus suggesting a strong antimicrobial potential of the plant (Ngwoke et al., 2014). Urinary tract infections (UTIs) are a serious public health problem affecting 150 million people worldwide (Stamm & Norrby, 2001). UTIs are commonly caused by several pathogens such as *Escherichia coli, Klebsiella pneumoniae, Enterococcus faecalis, Staphylococcus saprophyticus, Proteus mirabilis,* and *Staphylococcus aureus.* Some patients face recurrent UTIs and hence require repeated prescriptions of antibiotics. This potentially increases the antimicrobial resistance of the uro-pathogens, which in long term will greatly increase the economic burden for countering future infections (Flores-Mireles et al., 2015). As multidrug-resistant pathogens are a serious threat to the society, the antimicrobial activity of AM has been investigated as an alternative to antibiotics. Results show that AM is effective against some clinically important bacterial and fungal isolates such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus* sp., *Salmonella* sp., *Bacillus* sp., *Escherichia coli*, *Klebsiella* sp., *Saccharomyces* sp., *Aspergillus* and *Candida* (Odetunde et al., 2015).

Virtually every part of AM possesses different phytochemicals that exert antimicrobial activity, which can be extracted by a repertoire of solvents. For instance, ethanol can be used to extract various phytochemicals in the seeds and leaves of AM. The ethanolic leaf extract contains phytochemicals such as tannins, alkaloids, saponins, steroids, cardiacglycoside and

terpenoids that have remarkable therapeutic actions in the treatment of gastrointestinal infections, nausea, respiratory problems, colds, fever, allergies, urinary tract infections and fungal infections. In short, ethanolic extract of AM has a broad spectrum of antimicrobial activity.

In a separate study, AM was found to inhibit the growth of *Salmonella* spp. and *Shigella* spp. (Doherty et al., 2010). In the acetone extract of AM's rhizomes, the presence of labdane diterpenoids such as zerumin A (Figure 3A) and (E)-labda-8(17),12-diene-15,16-dial (Figure 3B) can effectively kill Gram-positive bacteria such as *Escherichia coli* and *Listeria monocytogenes*. The labdane diterpenoids also inhibit the growth of MRSA, which is known to be resistant to most antibacterial agents. Furthermore, both zerumin A and (E)-labda-8(17),12-diene-15,16-dial exhibit greater antibacterial activity towards MRSA compared to the antibiotics such as ampicillin, gentamicin and vancomycin that are currently used clinically. Thus, they may represent potential antibacterial lead compounds (Ngwoke et al., 2014). Aqueous extraction of AM's rhizomes for phytochemicals, however, is not as effective as acetone extraction. Hence, acetone is a more appropriate solvent to be used for the extraction of diterpenoids from the rhizome of the plant (Eloff, 1998).

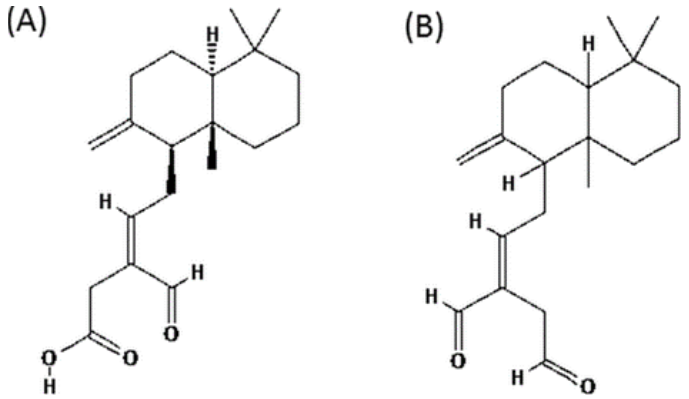

Figure 3. Chemical structures of (A) Zerumin A and (B) (E)-labda-8(17),12-diene-15,16-dial

Hepatoprotective Effect

The liver plays a vital role in metabolising toxic substances that enter the body, including alcohol, which is one of the major causes of liver diseases worldwide (Leggio & Lee, 2017). In the liver, alcohol is broken down into simpler end-products such as acetaldehyde to be easily eliminated from the body. Highly reactive molecules such as reactive oxygen species (ROS) generated during the metabolism of alcohol may contribute to the development of alcoholic liver diseases such as cirrhosis, simple steatosis and acute alcoholic hepatitis. The reactive molecules can destroy the vital cell components in the liver through

oxidation (Dunn & Shah, 2016; Fernández-Checa et al., 1997). Consequently, oxidative stress accumulates in cells from the imbalance between oxidants and antioxidants. Excessive oxidants damage the mitochondria, thus diminishing energy production (Fernández-Checa et al., 1997). Therefore, excessive alcohol consumption affects the health of the liver as well as that of other organs (Nordmann et al., 1992). Alcohol toxicity causes acute liver disease, and prolonged frequent consumption may lead to chronic liver diseases (Cederbaum et al., 2009).

On the other hand, chemical solvents such as carbon tetrachloride (CCl_4) used in the lab causes liver toxicity upon excessive exposure. CCl_4 can induce apoptosis of hepatocytes, and the oxidative stress and generation of free radicals may further harshen the CCl_4-induced liver injury (El-Halawany et al., 2014). Excessive alcohol consumption and inhalation of chemical solvents like CCl_4 can lead to significant elevation of serum alanine aminotransferases (ALT), aspartate aminotransferases and triglyceride levels, as well as a decrease in glutathione (GSH) and superoxide dismutase (Nwozo & Oyinloye, 2011). Tumour necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) levels are increased if the liver is inflamed (El-Halawany et al., 2014).

The humble seeds of the melegueta pepper have been proven to reverse such toxic effects. Its hepatoprotective effect is evaluated based on the changes in ALT, GSH, thiobarbituric acid reactive substances, TNF-α, IL-1β levels and activities of some enzymes such as caspase 3 and 9 (El-Halawany et al., 2014). Phytochemicals such as phenols and flavonoids that are present in the aqueous seed extract of AM were shown to have hepatoprotective effects (Nwozo & Oyinloye, 2011). These phytochemicals possess antioxidant properties which neutralise liver-damaging free radicals and reactive oxygen species (Choi et al., 2006). Methanolic and chloroform seed extracts of AM also exhibit similar effects. Compounds present in the extract include 3-(*S*)-acetyl-1-(4′-hydroxy-3′, 5′-dimethoxyphenyl)-7-(3″,4″,5″-trihydroxyphenyl) heptane and dihydrogingerenone (Figure 4) that can supress TNF-α and IL-1β levels in the body. These compounds impede the reduction of GSH by trapping free radicals arising from liver hepatocytes. In addition, these compounds also lower the levels of ALT, TNF-α, IL-1β as well as caspases 3 and 9. Apoptosis of hepatocytes induced by CCl_4 is also inhibited (El-Halawany et al., 2014). In short, the hepatoprotective effect of AM is due to its ability to suppress inflammatory responses and apoptosis, as well as the ability to forage free radicals.

Although significant in effect, the number of bioactive compounds found to contribute to the hepatoprotective effect in AM are quite limited in current literature. Hence, concerted efforts to extract and screen compounds from the plant for their biological activities and their effects should be expanded and its active metabolites tested *in vivo*. Different solvents might be used for extraction of different active compounds.

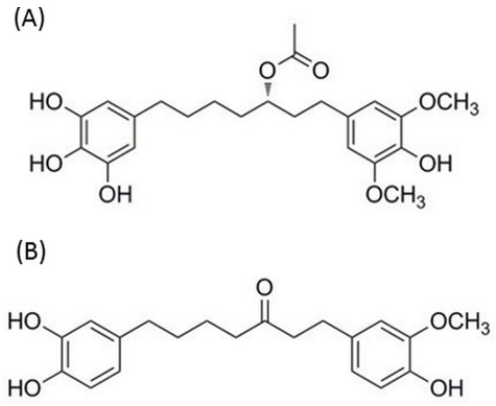

Figure 4. Chemical structures of (A) 3-(S)-acetyl-1-(4'-hydroxy-3',5'-dimethoxyphenyl)-7-(3",4",5"-trihydroxyphenyl) heptane and (B) Dihydrogingerenone

Anti-cancer Effect

The legendary cancer-battling antioxidants, flavonoids, have been found in relative abundance in AM (Doherty et al., 2010). Flavonoids are commonly reported to possess anti-carcinogenic and anti-mutagenic effects (Aranganathan & Nalini, 2013) in which they interfere with the development of malignant tumours by inhibiting the expression of mutant genes, inactivating carcinogens and enzymes that are involved in the activation of pro-carcinogens, as well as activating enzymatic systems that are involved in the detoxification of xenobiotics (Bravo, 1998). Flavonoids also inhibit the initiation, promotion and progression of tumours (Okwu, 2005; Urquiaga & Leighton, 2000). Quercetin, a flavonoid which can decelerate the development of tumours (Clifford et al., 1996), was also found to be present in the AM extract (Adefegha & Oboh, 2012). Although past studies have suggested that flavonoids isolated from various plants are effective against cancer cells, there is limited work on flavonoids isolated from AM. In a previous study, evidence showed that AM extracts were effective against pancreatic cancer (Dibwe et al., 2012).

Other supporting evidence includes a study by Kuete et al. (2011) who showed that the AM extract exerted significant inhibitory activities on human pancreatic cancer and leukaemia cell lines. Phytochemical investigations revealed the presence of (-)-buplerol, (-)-arctigenin, (E)-14-hydroxy-15-norlabda-8(17), 12-dien-16-al, labda-8(17),12-dien-15,16-dial, 16-oxo-8(17),12(E)-labdadien-15-oic acid, 5-hydroxy-7-methoxyflavone and apigenin in the extract. Among the list, (-)-arctigenin and (-)-buplerol showed the capacity to trigger apoptosis in pancreatic cancer cells (Dibwe et al., 2015).

The anti-cancer ability of AM may not be attributed to flavonoids alone. Paradols, common plant phenolic compounds, are also found to exert anti-cancer effects by inducing apoptosis in human pro-myelocytic leukaemia (HL-60) cells. The effect is due to the presence of a vanillyl moiety and ketone functional group in the compound. Additionally, paradols can also suppress tumour promotion of the skin *in vitro* (Chung et al., 2001).

Discovery of bioactive compounds that have anti-cancer potential from AM is very limited. Hence, the identification of novel anti-cancer compounds from AM and investigation on their mechanisms of action may be an area for further exploration.

Furthermore, utilisation of different solvents or extraction methods might be useful in isolating novel compounds from the plant.

Anti-diabetic Effect

In addition to its repertoire of therapeutic effects, AM may also prove its worth in combating metabolic disorders such as Type 2 diabetes. In this condition, the body resists the physiological effect of insulin. Therefore, too much insulin will remain in the blood for extended periods of time, causing the pancreas unable to secrete more insulin to control the glucose level in the blood. As a consequence, postprandial hyperglycaemia ensues (Gastaldelli, 2011).

A myriad of compounds found in AM such as 6-paradol, 6-shagaol, 6-gingerol, oleanolic acid and acarbose exert an anti-diabetic effect by inhibiting enzymes such as α-amylase and α-glucosidase. These enzymes are responsible for digestion and break down of the carbohydrates and polysaccharides from food into simple sugars to increase blood glucose levels. Among the compounds, 6-gingerol and oleanolic acid are more effective in inhibiting the enzymes (Mohammed et al., 2017). Based on the current evidence, AM is suitable for consumption by diabetic patients.

Anti-inflammatory Effect

Fascinatingly, AM extract has been known to reduce fat and even relieve painful arthritis when it is used as a massage oil (Odetunde et al., 2015). Meanwhile, the ethanolic seed extract contains phytochemicals such as tannins, saponins, flavonoids, steroids, terpernoids, cardiac glycosides and alkaloids that possess antimicrobial and anti-inflammatory effects (Doherty et al., 2010; Okwu, 2004). Okoli et al. (2007) provided evidence that the methanolic AM extract and its fraction contained alkaloids, glycosides, tannins, flavonoids, sterols and resins, with alkaloids and tannins as the major compounds. Both the extract and fraction showed potential systemic anti-inflammatory activity, as they inhibited rat paw oedema induced by egg albumin.

To support this claim, Ilic et al. (2014) reported that the ethanolic AM extract inhibited cyclooxygenase-2 (COX-2). Compounds that inhibit COX-2 activity are capable of reducing inflammatory responses (Seibert & Masferrer, 1994). The most active COX-2 inhibitory compound in the AM extract was [6]-paradol, while [6]-shogaol was found to inhibit expression of a pro-inflammatory gene, interleukin-1 beta (IL-1β). Results from the paw oedema model showed that the AM crude extract and its active compounds [6]-paradol, [6]-gingerol and [6]-shogaol significantly reduced inflammation in rats.

In another study utilising the aqueous seed extract of AM, sub-chronic inflammation was induced by 2% formaldehyde or 6% nystatin, while chronic inflammation was induced by carrageenan in rats. The results revealed that AM extract significantly reduced oedema induced by formaldehye and nystatin. Furthermore, it reduced the exudate induced by carrageenan (Umukoro & Ashorobi, 2005). El-Halawany et al.

(2014) suggested that the anti-inflammatory potential of AM might be due to its ability to downregulate cytokines such as TNF-α and IL-1β.

The present studies utilised ethanol, methanol and water for the extraction of compounds from AM. The role of hexane, ethyl acetate, and other alternative solvents is worth exploring as it may yield yet-to-be discovered active compounds from the plant.

Haematopoietic Effect

Another lesser-known finding is the influence of AM on blood cell production. When the methanolic seed extract of AM was administered to 2,4-dinitrophenylhydrazine-induced anaemic rats, the treatment showed an increase in haemoglobin levels and platelet count, indicating its erythropoietic potential in treating anaemia (Omoboyowa et al., 2017). Paradoxically, higher doses of the extract have previously been observed to be haematotoxic (Akpanabiatu et al., 2013), and thus the administered dose should be selected with caution.

CONCLUSION

Based on available evidence, AM consists of several phytochemicals that exert beneficial pharmacological activities. For instance, the presence of flavonoids, tannins, alkaloids, saponins, steroids, cardiac glycosides, terpenoids, labdane diterpenoids such as zerumin A and (E)-labda-8(17),12-diene-15,16-dial exhibit significant antimicrobial effects, with some even more effective than antibiotics. Moreover, compounds such as 3-(*S*)-acetyl-1-(4′-hydroxy-3′,5′-dimethoxyphenyl)-7-(3″,4″,5″-trihydroxyphenyl) heptane and dihydrogingerenone demonstrate hepatoprotective effects. Furthermore, AM phytochemicals such as flavonoids, paradols and phenolic compounds exert anti-cancer effects on oral squamous carcinoma and pro-myelocytic leukaemia. Finally, compounds such as 6-paradol, 6-shagaol, 6-gingerol, oleanolic acid and acarbose exhibit anti-diabetic effects by inhibiting the enzymes that metabolise carbohydrates into glucose. In summary, AM harbours an increasing inventory of health benefits and may serve as a source of potential alternative medicines for various disease states.

Despite its vast potential, current literature on the pharmacological effects of AM is very limited. Therefore, detailed studies on the various anatomical parts of AM could be systemically performed, and specific ranges of solvents or extraction methods could be used to maximise the mining of bioactive compounds from the plant. In addition, its effects on other biological activities such as immunomodulation could be explored, as AM is rich in phenolic compounds that may potentially contribute to such effect. More importantly, toxicological studies should be implemented prior to enlisting AM as a possible source of therapeutic drugs.

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Changes in Flour Quality of Four Iraqi Wheat Varieties During Storage

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ABSTRACT

The issue related to suitable wheat age after harvesting for producing flour is raised regularly during a harvest season. Therefore, determining the suitable wheat storage for different wheat strength and with high extraction rate (80%) is necessary to produce a good quality flour. Four varieties of bread wheat (Adina, Aibaa99, Sham6, and Rasheed) were tested. Gluten amount, gluten index, amylase activity, dough rheological properties (farinograph parameters), and flour size were detected. Results showed that gluten index was stable for Adina variety (the strongest variety), but it was reduced significantly during week 11th and 15th for Sham6 and Aibaa99 (weak wheat varieties) respectively. The α-amylase activity of all varieties was reduced during storage periods. Dough stability increased during storage period for all four varieties. However, the increment was higher in Adina compared to others (lower strength wheat). Mixing tolerance index (MTI) of Adina was changed slightly during the storage period while for other varieties MTI increased sufficiently. In conclusion, wheat varieties behaved differently depending on wheat strength, and there was no specific time that all that dough properties improved during the study period.

Keywords: Dough quality, rheological properties, wheat storage

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INTRODUCTION

Cereal is an important crop all over the world because of its nutritional value and low price. Cereals provide high starch content, energy source, dietary fiber, protein, lipid that rich in essential fatty acids, and micronutrients (Dewettinck et al., 2008). Wheat is considered one of the most important crops in the world (Shewry et al.,

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2002). The quality of wheat flour for bread making is generally determined by content and type of its protein, starch conditioned (damage) and enzymes. These factors are controlled by wheat variety, time and conditions of wheat harvest, and milling technology (Hrušková & Machová, 2002) in addition to wheat and flours ages (Mense & Faubion, 2017). After wheat harvest and before milling, wheat moisture changes occur, which is considered the main factor affecting flour quality in term of wheat age (Posner & Deyoe, 1986).

Flour quality can be determined by analyzing chemical and rheological properties of its dough. Quality and quantity of gluten (wheat protein) are one of the most important properties of flour in producing bread because of dough extension (gas retention) that can be determined by the amount and type of gluten (Hadnađev et al., 2011). Gluten index is considered a quicker method to determine wheat quality (weak, normal, or strong) compared with other methods such as fariongraph or mixing graph (Oikonomou et al., 2015). Another important device to determine dough physical properties is farinograph that can determine water absorption, mixing tolerance index, stability, and development time. These properties are useful in term of optimizing baking quality (Yazar et al., 2016). Starch gelatinization can be determined indirectly by measuring falling number. Falling number (FN) indicates total α-amylase in the grain. The higher FN, the less α-amylase activity. High level of α-amylase reduces baking quality, which can be indicated by FN less than 250s (Ral et al., 2016).

The issue related to the suitable wheat age for flour production is raised regularly worldwide during harvested seasons. In Iraq, Grain Board of Iraq (GBI) imports raw wheat (usually stored for several months) continuously that is mixed with the demotic wheat, sometimes the imported wheat is not available because of some political or technical issues. In this situation, GBI entirely depends on the cultivated wheat in Iraq, especially during the harvest season (May to August). Therefore, the aim of the study is to determine the suitable wheat ages of different Iraqi wheat varieties (strong and weak wheat) to produce high quality flour with high extraction rate (80%), which is used in Iraq.

MATERIALS AND METHODS

Four varieties of Iraqi wheat named Adina, Aibaa99, Sham6, and Rasheed (commonly varieties in Iraq) were harvested from farms of Kirkuk (north Iraq) on 20th and 21st of June 2017 and stored at ambient temperature until 26th of November (about five months). The varieties identity confirmed by the Ministry of Agriculture members that they had provided seeds to the farmers. Flour was produced and analyzed for the first week, third, fifth, seventh, 11th, 15th, 19th, and 23rd week of storage. Flour was produced after wheat was cleaned from impurities, moisturized to 16% moisture for 20-24 h, and milled by using Quadermet miller (Brabender® OHG, Brabender GmbH Co. KG, Duisburg, Germany) to 80% extraction

rate (rate used in Iraq mills). The produced flour was analyzed within two days from its production to avoid flour age interferences for ash (AACC Method 08-01), moisture (AACC Method 44-10), wet gluten and gluten index (AACC Method 38-12), farinograph (AACC Method 54-21), particle size by using Buhler Laboratory Siftermin 300 (Bühler Group Company, Uzwil, Switzerland) for five minutes, protein (AACC Method 39-01), and falling number (56-81B) (American Association of Cereal Chemists [AACC], 2000). Farinograph analysis of week five was not detected because of some technical issues, and the analyzed had not been detected a week after because of flour storage issue that would interfere with the result.

Statistical Analysis

One-way analysis of variance (ANOVA) was performed for statistical analysis of data. Least Significant Difference (LSD) of means was implemented by using SAS version 9.0 (Cary, NC, USA). Significant differences were considered at $\alpha = 0.05$ level. All treatments were conducted in duplicate.

RESULTS AND DISCUSSION

In this study, the effect of wheat storage on the produced flour of the four wheat varieties cultivated in Iraqi was examined in terms of chemical and quality properties of dough. There were differences in wheat varieties used in this study in term of protein content, gluten quality and quantity, and rheological properties (farinograph) of dough produced from the wheat varieties. Using different wheat varieties (different quality) were important to know how each variety changed during the storage time. Table 1 indicates protein, ash, and moisture content of whole flour (100% wheat flour) and produced flour (first week) of the four varieties of wheat. For whole wheat flour, Aibaa99 variety had the highest protein content (12.1% at 14% mb), while Sham6 varieties had the lowest amount of protein (9.9% at 14% mb). The quantity and quality of gluten of the first week were shown in Table 2. While Aibaa 99 still had the highest amount of gluten (31.6%) for the first week compared with all other types, the quality of gluten determined by gluten index (76.5%) refers to the lowest quality compared with the other varieties for the first week. Increasing gluten index refers to the gluten strength (Hadnađev et al., 2011). All four tested wheat varieties had low amylase activity for the first week, and the highest activity was noticed in Sham6 varieties, which had falling number 399s (Table 2). The best falling number range occurs between 250-300s (Ral et al., 2016).

Table 2 indicates wet gluten (%), gluten index (%), and falling number (s) of flour produced from the four varieties of wheat for the first week to the 23rd-week ages. Increasing gluten amount and high gluten index (Meerts et al., 2017) are a good indication of high-quality dough. While the variables fluctuated during the storage period, it is necessary to highlight some significant differences during the storage to explain the results in a useful way. Wet

Table 1

Whole wheat flour and produced flour characteristics (1st week) of the four wheat varieties

Characteristic	Wheat Types			
	Adina	Aibaa99	Sham6	Rasheed
Whole wheat Flour*				
Protein (14%mb)**	11.1 ± 0.1	12.1 ± 0.1	9.9 ± 0.1	11.3 ± 0.0
Ash (14%mb)	1.41 ± 0.01	1.57 ± 0.01	1.43 ± 0.01	1.69 ± 0.00
Moisture%	7.6 ± 0.6	7.6 ± 0.4	7.4 ± 0.4	7.6 ± 0.3
Produced Flour***				
Protein (14%mb)	10.6 ± 0.3	11.5 ± 0.4	9.4 ± 0.6	11.0 ± 0.4
Ash (14%mb)	0.81 ± 0.01	0.89 ± 0.03	0.82 ± 0.02	0.93 ± 0.02
Moisture%	14.6 ± 0.3	14.7 ± 0.3	14.4 ± 0.5	14.7 ±0.1

*Whole flour is produced from milling whole wheat (100%) without removing the bran
**mb (Moisture basis)
***Produced flour is flour with 80% extraction rate

gluten of Adina was reduced during the storage period. It was reduced significantly from 27% for the first week to 23.7% for week 23rd and this result agreed with the Posner and Deyoe (1986) study that wet gluten was reduced from 27.4 to 24.8%. In their study, they moisturized wheat to 16% before milling and used freshly harvested hard red winter. Wet gluten percentage for Aibaa99 was reduced significantly during week 11th and 15th compared to week 19th and 23rd, while there were no significant differences of wet gluten content for Sham6 and Rasheed varieties during the storage period. Gluten index percentage of Adina was almost stable during storage period except during week 19th was reduced significantly (Table 2). Gluten index of Aibaa99 variety (the lowest gluten index in the first week) had been improved from 76.5% to the highest value (98.4%) during week 19th, which indicated that storage time had a positive effect on gluten index of a weak wheat variety. For Sham6 variety, gluten index reduced significantly only during week 11th to 71.5% compared to 93% of the first week. Gluten index of Rasheed variety was reduced during 5th to 11th week storage, however, the reduction was not significant. The fluctuation in gluten index for all varieties during the storage period probably refers to some protein chemical bonds changes during storage. Further study is required to determine what protein bonds are formed or broken to cause these changes and to determine what the reason behind the fluctuated results was. Variation of protein and gluten of the produced flour during storage time is probably because of using the high extraction rate flour (80%) that have some bran, which led to producing different particle size during the storage period

Table 2
Produced flour properties during storage time of the four varieties (Adina, Aibaa99, Sham6, and Rasheed)

	Adina				Aibaa99			
Time after Harvest (W)	Protein% (14%mb)	Wet Gluten% (14%mb)	Gluten Index%	Falling Number (S)	Protein% (14%mb)	Wet Gluten% (14%mb)	Gluten Index %	Falling Number (s)
1	10.6	27.04 ± 0.78a	99.00 ± 0.85a	431 ± 2d	11.5	31.61 ± 2.50a	76.50 ± 0.71b	441 ± 41d
3	9.9	26.75 ± 1.37a	100.00 ± 0.00a	451 ± 8d	11.0	30.65 ± 2.66ab	75.00 ± 0.00b	494 ± 13bc
5	10.2	24.73 ± 0.71ab	99.15 ± 1.20a	434 ± 1d	10.8	29.22 ± 0.35ab	74.00 ± 14.14b	529 ± 9b
7	10.1	26.45 ± 0.65a	99.25 ± 1.10a	445 ± 6d	11.0	30.7 ± 1.44ab	68.00 ± 7.07b	532 ± 4b
11	10.0	26.36 ± 0.29a	100.00 ± 0.00a	625 ± 1a	10.9	26.28 ± 0.79b	82.50 ± 2.12ab	621 ± 2a
15	10.0	25.12 ± 0.57ab	100.00 ± 0.00a	524 ± 0bc	10.8	28.24 ± 24b	77.50 ± 0.71b	432 ± 2d
19	9.9	25.02 ± 0.00ab	94.90 ± 0.28b	547 ± 6b	10.8	33.16 ± 0.0a	98.35 ± 0.00a	459 ± 39cd
23	9.8	23.67 ± 0.73b	99.35 ± 0.92a	504 ± 7c	10.8	29.06 ± 0.80a	90.60 ± 1.56ab	494 ± 6bc
Sham6					Rasheed			
1	9.0	23.21 ± 1.85a	93.00 ± 5.70a	399 ± 1cd	11.0	29.29 ± 0.07a	96.00 ± 0.00ab	474 ± 6c
3	9.1	24.44 ± 2.44a	95.50 ± 3.50a	397 ± 26cd	10.5	28.9 ± 0.43a	90.00 ± 0.00ab	511 ± 23bc
5	9.1	22.71 ± 0.14a	89.50 ± 3.50a	414 ± 60bc	10.7	29.09 ± 1.36a	86.50 ± 4.95b	471 ±6c
7	9.1	22.39 ± 0.50a	91.50 ± 5.00a	399 ± 2cd	10.7	29.14 ± 1.29a	87.50 ± 6.36b	535 ±11b
11	9.2	23.43 ± 0.36a	71.50 ±9.20b	318 ± 1d	10.6	27.77 ± 3.52a	88.00 ± 4.24b	796 ±45a
15	9.1	23.95 ± 1.00a	87.50 ± 5.00a	491 ± 1ab	10.6	28.06 ± 0.29a	98.50 ± 0.71a	489 ± 1bc
19	9.1	26.95 ± 0.07a	97.30 ± 0.60a	518 ± 40ab	11.0	30.88 ± 0.22a	95.85 ± 1.20ab	498 ± 0bc
23	9.2	22.78 ± 0.00a	97.50 ± 0.30a	492 ± 7a	11.0	29.29 ± 0.07a	96.00 ± 0.00ab	474 ± 6c

Values are expressed as a mean ± SD from two independent experiments. Means with different letters within the same column are significantly different at $p<0.05$

(Figure 1). Noort et al. (2010) mentioned that adding bran to a bread flour had a negative effect on the bread quality because of fiber-gluten interaction. In addition to increasing extraction rate of flour which lead to increase bran and germ, leading to increase in protein, fat, minerals, vitamin B, and fiber. However, baking quality decreases (Mense & Faubion, 2017).

Falling number values of flour produced from wheat stored for different times generally increased during storage (reduce amylase activity). For Adina, Aibaa99, and Rasheed, the highest falling number was at 11th week, while for Sham 6 variety was increased maximally during week 19th, however, there were no significant differences between week 19th and weeks 5th, 15th, and 23rd. Increasing falling number refers to reduced α-amylase activity. The best falling number for bread flour occurs between 250-300s (Polat & Yagdi, 2017). Mense and Faubion (2017) reported that falling number of a wheat variety increased

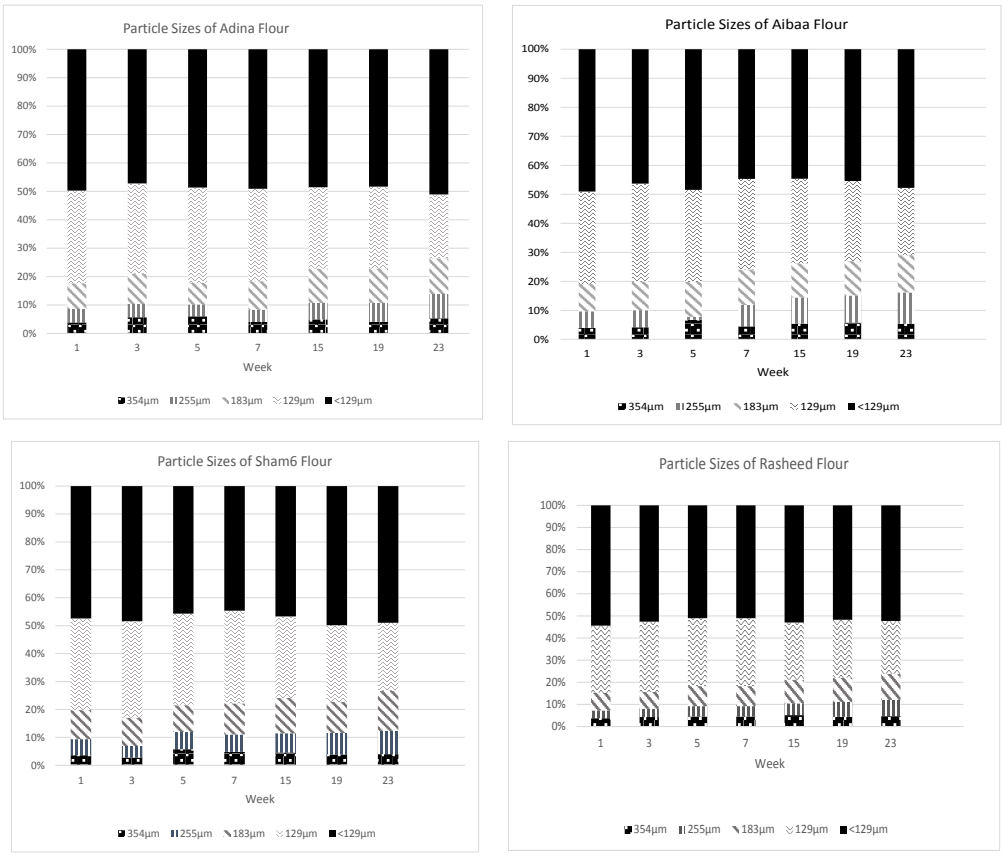

Figure 1. Particles sizes of produced flour of the four varieties during storage time. The mentioned particles were above the sieves except for <129, which was under the sieve

to 439s after 13 weeks storage time compared to 418s at the first week, while the other tested variety in the same study was almost stable during the same storage period. However, the increment was higher in our study compared with the Mense and Faubion (2017) study. The α-Amylase activity of the tested varieties at the first week was less than the optimum range. During storage, α-amylase was further reduced, therefore α-amylase was affected negatively by increasing storage time.

Table 3 demonstrates farinograph data of flour produced from the four wheat varieties during 23 weeks of storage period. Increasing stability, development time, water absorption, and reducing MTI are a good indication of high-quality dough for bread production (Wahyono et al., 2016). Water absorption of flour produced from Adina, Aibaa99, and Sham6 varieties were changed during the storage period, but mostly they were little higher than the first week. For Rasheed variety, water absorption was almost stable during the first eleven weeks, but it was reduced during week 15th and increased again during last week. Water absorption value is considered the most important value of the farinograph parameters, and it directly refers to finished bakery products volume (Hadnađev et al. 2011; Okuda et al., 2016). Diósi et al. (2015) mentioned a classification of flour depending on the fariongraph parameters, which were the A quality group should have a minimum 60.0% water absorption, and the B quality group should have a minimum 55.0% water absorption with a minimum 10.0 min and 6.0 min stability respectively. Vetrimani et al. (2005) reported that there was a positive correlation between the extraction rate and water absorption for vermicelli dough. Water absorption of all varieties increased little during storage time and that refered to little enhancing of dough quality in this term (Ahmed et al., 2017). Development time of four varieties either reduced or was stable during the storage period. Development time of Adina variety was stable during storage period except during week 7th and 11th reduced to 5.3 and 5min compared to 6min. In Aibaa99 variety, development time was different during the storage period, the minimum was in week 7th (3.3min) and the maximum was in the week 19th and 23rd (4min). Development time of Sham6 variety was reduced maximum during week 11th and increased again after that. In Rasheed variety, development time was reduced during and after week 15th. Reducing development time during storage period refers to decline dough properties. Dough stability of produced flour was generally increased during wheat storage. The highest increment of stability was in Adina, stability was increased from 6.8min during the first week to the maximum stability in the last week (10min) (Table 3). For Aibaa99 and Rasheed varieties, stability increased from 3.2min and 4 min in the first week to the highest stability (5.5 min and 6 min) in the week 11th respectively. Dough stability of Sham6 variety was increased during storage period to maximum (5min) at the last week compared to the minimum at the first week (3min) (Table 3). Mixing tolerance index

Table 3
Farinograph characteristics of the four varieties during storage period

Time after Harvest (W)	Water Absorption %				Development Time (min)			
	Adina	Aibaa99	Sham6	Rasheed	Adina	Aibaa99	Sham6	Rasheed
1	58.5	58.2	57.7	58.3	6.0	3.8	4.2	4.0
3	60.4	59.6	59.2	58.7	6.0	3.6	3.2	4.0
7	58.2	58.6	58.4	58.4	5.3	3.3	3.5	4.0
11	59.3	59.1	59.4	58.9	5.0	3.5	3.0	4.0
15	59.2	58.3	57.9	56.2	6.0	3.5	3.5	3.5
19	59.3	59.2	57.6	58.7	6.0	4.0	3.5	3.5
23	59.4	60.1	58.3	59.8	6.0	4.0	3.5	3.5

Time after Harvest (W)	Stability (min)				MTI (BU)			
	Adina	Aibaa99	Sham6	Rasheed	Adina	Aibaa99	Sham6	Rasheed
1	6.8	3.2	3.0	4.0	40	40	61	63
3	8.2	4.1	3.0	4.2	34	67	88	61
7	9.0	4.2	3.5	4.5	33	56	78	44
11	9.0	5.5	4.0	6.0	44	44	89	44
15	8.5	5.0	4.5	4.5	44	77	67	67
19	7.0	5.0	4.0	5.0	45	89	89	89
23	10	5.0	5.0	5.0	33	56	56	55

(MTI) of flour produced from Adina variety was reduced to minimum Brabender Unit (BU) during week 7th and week 23rd. For Aibaa99 variety, the highest MTI was 89 BU during week 19th compared to 40 BU in the first week. Storage time had no reducing effect on MTI of the flour produced from Aibaa99 variety. For Sham6 variety, MTI increased to the highest value during 11th and 19th weeks, while reduced to lowest value during 23rd week. For Rasheed variety, MTI reduced during storage period except during week 15th and 19th. Adina variety was faster (7 weeks) than other varieties in term of increasing dough stability and little alterations of MTI. Hadnađev et al. (2011) mentioned that high stability and lower MTI refered to more dough tolerance to mixing. Stability of all other varieties was increased, but the increase was less than in Adina variety, which was the strongest one. This result probably indicates that storage period effect positively on the strong wheat more than on the weak wheat. Although, Adina is considered the strongest variety compared to the tested varieties depending on gluten index value (99%), water absorption (58.5%), stability (6.8 min), and MTI (40 BU) of the first week (Tables 2 and 3), it is a B quality group depending on the Diósi et al (2015) classification that was mentioned above. Adina variety was the only variety that reached 10 min stability after 23rd-week storage (Table 3). The other varieties had not met the B quality properties after 23rd storage period except for Rasheed variety during week 11th (Table 3). Wheat cultivated in Iraq depending on the varieties tested in this study needs quality improvement to meet quality A group or at least quality B.

Figure 1 shows particle sizes of produced flour of the four varieties, and it indicates that the particle sizes were different in different storage time of each variety. The main change in particle size during storage time was reducing of particle size between 129 and 183 µm. Water absorption has a correlation with extraction rate. Particle size of flour influences the rheological properties of dough. Bressiani et al. (2017) mentioned that particle size influenced the functionality of the gluten network and consequently the bread volume. Reduced particle size of flour leads to increase starch damage (break starch to small particle sizes) and increase surface area that causes more dough absorption and consequently more dough stickiness (Gaines, 1985). Further, increasing extraction rate of flour to 80% probably implies some bran layers in the producing flour (Mense & Faubion, 2017). The effect of adding different particle sizes of bran to flour was reported in literature contradictory (Noort et al., 2010). Bressiani et al. (2017) mentioned that reducing particle size of bran enhanced bread volume. While Noort et al. (2010) stated that reducing particle size of bran had negative effect on baking. However, in our study, the alteration of particles size occurred naturally during storage period although the producing method was same in all period intervals. This probably indicates that wheat storage has an effect on the milling process that leads to form different particle size of the producing flour.

CONCLUSION

The results emphasize that the tested wheat varieties were not strong enough either at the first week or after 23rd week storage. Iraqi wheat varieties need to be improved to meet A quality group of farinograph parameters. A wheat variety is considered an important factor in determining the suitable wheat age to produce flour. The stronger wheat variety, the faster changes occurred. Probably because all the tested wheat varieties were not strong enough, therefore, no clear improvement occurred. There was no specific storage period that all the dough enhanced at the same time. Some properties were improved, declined, or had no clear effect.

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Elateriospermum tapos Seed Protein as a New Potential Therapeutic for Diabetes, Obesity and Hypertension: Extraction and Characterization of Protein

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ABSTRACT

The *Elateriospermum tapos* seed extract is one of the major contributors to the therapeutic effects as remedy drink for chronic diseases, such as hypertension, diabetes and obesity by the locals in East Malaysia. It is believed that the component in the seed, such as protein, plays an essential role due to its nutraceutical properties. Hence, we report the extraction of protein from the seed of *E. tapos* that has the potential biological activities. The protein was initially extracted using salt solution, which was similar to the local practice and maximal values of extraction yield (44 mg/g) was performed based on the extraction parameters; pH 8, sample to buffer ratio, 1:50 (w/v), 2 h at 80 °C. Sodium dedocyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) profile showed that 7S globulin was the dominant protein in the *E. tapos* seed protein isolate (ETSPI), whereas Fourier Transform Infrared (FTIR) analysis showed that the extracted protein consisted of α-helical secondary structure. This protein could be digested up to 72.5% *in vitro*. In the biological activity evaluation, the results showed that ETSPI exhibited angiotensin converting enzyme (ACE) inhibitory (56.6%/100 µg), lipase inhibitory (26.7%/100 µg), α-amylase inhibitory (31.2%/100 µg), free radical scavenging (33.4%/mg) and metal reducing activities (0.97 mM $FeSO_4$/mg), which suggests that this protein has the potential in the aforementioned therapeutic effects.

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INTRODUCTION

Elateriospermun tapos (*E. tapos*), locally known as *kelampai* or *buah perah*, is an

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indigenous palatable seed commonly found in Borneo, Peninsular Malaysia, Thailand as well in the Sumatra tropical rainforest (Van Sam & Van Welzen, 2004). This monoecious canopy species originated from the family of Euphorbiaceace and subfamily of Crotonoideae. It was reported that the fresh latex from *E. tapos* can be utilized traditionally as a wound healing agent (Corner, 1988). Apart from that, *E. tapos* was reported to be a good source of edible oil due to the extensive high amount of α-linolenic acid content (Yong & Salimon, 2006). In addition, the seed flour of the *E. tapos* was found to exhibit a good emulsifying property, which could be potentially used as a food formulation agent (Choonhahirun, 2010). The seeds were also popular among the locals as they believed that aqueous extract (as a traditional medicine drink) from *E. tapos* seeds have the ability in treating chronic diseases, such as hypertension. Even though there are many researches on the phytochemical compositions and nutritional value of *E. tapos*, however, the protein in the seed, which could also be present in the drink as aforementioned and play a major role in the therapeutic properties, has not been investigated. Storage proteins (e.g. globulin, glutemin, prolamin), α-amylase inhibitor, lectins and Bowman-Birk-inhibitors were some examples of the documented legumes derived bioactive protein, which had the potential in lowering the blood glucose and blood pressure. The importance of the pharmacological properties that implied on plant protein had lead the researchers in further investigating its roles, notably isolated from seed in reducing the risk of certain diseases. For instance, the protein isolated from Canary seed (Valverde et al., 2017) and Amaranth seed (Fritz et al., 2011) were reported to have the ability in reducing hypertension. Besides, many researches on seed protein had proclaimed that seed protein could be an antioxidant agent, such as canary seed protein (Valverde et al., 2017), canola seed protein (Cumby et al., 2008) and watermelon seed protein (Dash & Ghosh, 2017). Hence, the scope of our research was to be mainly focusing on finding the best extraction parameter of the protein as well as evaluating the biological activities (i.e. antidiabetic, antihypertensive, antiobesity and antioxidant activities) of the isolated protein from the local medicinal drink.

MATERIALS AND METHODS

Chemicals

All the enzymes (i.e. α-amylase (A3176), lipase (L3126) and angiotensin converting enzyme, (A6778)) used in the experiment were purchased from Sigma-Aldrich Company, Malaysia. The chemicals and reagents were used in the experiment were of analytical grade and purchased from Sigma-Aldrich, Malaysia, unless stated otherwise in the method.

Materials

Elateriospermun tapos fruits were purchased from Market Satok, located in Kuching, Sarawak. The seed of the fruit was separated

and washed with deionized water before lyophilised using Labconco Freeze dryer (Fisher Scientific, USA). Subsequently, blender was used to grind the dried seed into powders, and they were then sieved (30 mesh). The seed powders were defatted by using n-hexane before being proceeded with the extraction process.

Extraction of *E. tapos* Seed Protein Isolate (ETSPI)

Extraction of ETSPI was conducted according to method by Adebowale et al. (2007) with slightly modified and the ETSPIs were extracted in three independent batches as replicates. Generally, the defatted seed powder (1 g) was suspended in 0.2 M sodium phosphate buffer (Bendosen Laboratory Chemicals, Malaysia) at different pH (i.e. 6-8) and different sample to buffer ratio (i.e. 1:10 – 1:50, w/v). Subsequently, the solution was incubated at the designated temperature (i.e. 40-80^{o}C) and time (i.e. 0.5-2.5 h) at a constant orbital shaking speed of 250 rpm. The resulting slurry from the extraction was then centrifuged for 30 min at constant speed of 4500 rpm and the supernatant was collected and being determined the protein content by using the Bradford assay (Bradford, 1976). The extraction yield was determined based on the Bradford analysis, and expressed in mg protein per gram of sample.

Experimental Design

The single factor experiment was conducted in determining the best condition for extracting the free protein. A total of four parameters, namely, pH, temperature, sample to buffer ratio and extraction time were investigated on which one of the parameter was changed while the other parameters were kept constant.

For the effect of pH, pH ranging from 6 to 8 with interval of 0.5 was studied, whereas the temperature, time and sample to buffer ratio were kept constant at 60 ^{o}C, 1.5 h and (1:30 , w/v), respectively.

For the effect of time, extraction period ranging from 0.5 to 2.5 h with interval of 0.5 was studied, whereas the temperature, pH and sample to buffer ratio were kept constant at 60 ^{o}C, pH 7 and (1:30, w/v), respectively.

For the effect of temperature, temperature ranging from 40 to 80 ^{o}C with interval of 10 ^{o}C was studied, whereas the pH, time and sample to buffer ratio were kept constant at pH 7, 1.5 h and (1:30, w/v), respectively.

For the effect of sample to buffer ratio, the ratio at 1:10, 1:20, 1:30, 1:40 and 1:50 (w/v) were studied, whereas the temperature, time and pH were kept constant at 60 ^{o}C, 1.5 h and pH 7, respectively.

Characterization of ETSPI

The ETSPI was prepared according to aforementioned extraction procedure at the best condition and the protein was precipitated by using 0.1 M HCl through the adjustment of pH to the 4.5. The precipitated was then centrifuged at speed of 4500 rpm for 30 min, lyophilized and stored at 4^{o}C prior to analysis.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The ETSPI was identified by performing SDS-PAGE analysis under reducing conditions with used of 12% resolving gel and 4% stacking gel based on method of Laemmli (1970). All the SDS-PAGE reagents were purchased from Bio-Rad (U.S.A). Precision plus protein standard marker (10-250 kD) was used as the standard marker for this SDS-PAGE analysis.

Fourier Transform Infrared (FTIR) Spectroscopy

ETSPI lyophilized powder was desiccated prior to FTIR analysis. The FTIR spectra of samples were recorded from 500 to 4000 cm^{-1} using Nicolet Avatar 360 FT-IR E.S.P spectrometer with attenuated total reflectance (ATR) system (Agilent Technologies, Santa Clara, USA). Background scan was conducted prior to the sample scan. Isopropanol was used to clean the sample cell before loading a new sample. The resolution was 4 cm^{-1}, and iterations were performed 32 times. The scan speed was set at 5 kHz and sensitivity of 1. The spectra were analyzed using Resolution Pro version 5.2.0 software.

***In vitro* Digestibility of ETSPI**

In vitro digestibility of ETSPI was determined according to method as described by Hsu et al. (1977). In general, ETSPI solution (6.25 mg/ml) was prepared and the pH of sample was adjusted to 8 using NaOH. The multi-enzyme mixture (i.e. trypsin, chymotrypsin and pepsin) were immediately added to the sample and subjected to a continuous stirring at 37 °C for 10 min. The pH was recorded for every 1 min during the 10 min digestion period. The percentage of digestibility was calculated using the equation below:

$$\% \text{ Digestibility} = 210.46 - 18.10X \quad \text{(eq. 1)}$$

where, X is the pH of the sample after 10 min of digestion.

Amino Acid Composition of ETSPI

The ETSPI (0.1 g) was hydrolyzed at 110 °C for 24 h with 6 M HCl under seal condition. The sample was then added with 400 µl of 50 µmole/ml of L-α-amino-n-butyric acid (AABA, internal standard) and topped up to 100 ml using distilled deionized water. Subsequently, the sample was analyzed using a Waters-HPLC-System (U.S.A) with Waters 1525 Binary Pump, Waters 717 plus Autosampler, and Waters 2475 Mutli λ Fluorescence Detector (Tan et al., 2014). Waters AccQ·Tag™ Amino Acid Analysis Column (3.9 mm × 150 mm; packing material: silica based bonded with C_{18}) was used and maintained at 37 °C during analysis. Fluorescence was measured at wavelength of 250 nm for excitation and 395 nm for emission. Control of the apparatus and solvent mixing as well as plotting and evaluating were carried out using Breeze Workstation version 3.20. Two mobile phases were used: (a) AccQ·Tag™ Eluent A; (b) Acetonitrile/Water (60%/40%), and amino acids standard H (PIERCE, U.S.) was used as reference to calibrate the

HPLC-system. Methionine and cysteine were analyzed separately using performic acid procedure (Moore, 1963). Briefly, the sample (0.1 g) was mixed with 2 ml of performic acid solution (prepared by mixing 0.2 ml of 30% H_2O_2 with 1.8 ml of 88% formic acid) in an ice bath (0 °C) for 4 h, and subsequently, 0.3 ml of 48% hydrogen bromine (Merck, Germany) was added to the mixture in ice bath. In order to remove the bromine in the solution, 20 ml of 1 N NaOH (Mallinckrodt, U.S.A.) was added to the mixture and the resulting mixture was dried at 40 °C for 30 min. The sample was then hydrolyzed using 3 ml of 6 N HCl in an oven at 110 °C for 18 h. The HCl was then removed using rotary evaporator and the residue was dissolved in 5 ml buffer (pH 2) prior to analysis using the same setting. The amino acid composition of the ETSPI sample was presented as per 1000 residues.

In vitro Antioxidant Activities Determination

The antioxidant activity of ETSPI was performed using 2-2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) assay as described by Cai et al. (2004). The ABTS reagent was firstly incubated in the dark for 16 h at room temperature. The reagent was then diluted using ethanol to obtain an absorbance reading of 0.700 ± 0.005 at 734 nm. Subsequently, the diluted ABTS solution (3 ml) was mixed with ETSPI (5 mg) and incubated in dark for 6 min at 23 °C. The absorbance of mixtures was recorded at 734 nm. The ABTS scavenging activity was calculated using the equation below:

$$\text{ABTS scavenging radical activity} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \quad \text{(eq. 2)}$$

where $A_{control}$ was referred to absorbance of control at 734 nm while A_{sample} was referred to absorbance of sample at 734 nm.

Ferric reducing antioxidant power (FRAP) analysis was conducted according to the method of Benzie and Strain (1999). The FRAP reagent (3 ml) was pre-warmed for 30 min at 37 °C before added to the ETSPI sample (5 mg). Subsequently, the sample mixture was vortexed and incubated for 1 h at 37 °C. The absorbance of resulting mixture was measured at 593 nm using a spectrophotometer (Spectamax M5, Molecular Devices, USA). Iron (II) sulphate heptahydrate ($FeSO_4.7H_2O$) was used as a standard, and the result was expressed as mM $FeSO_4$ per gram of sample.

In vitro α-amylase Inhibitory Assay of ETSPI

The α-amylase inhibitory assay was carried out based on the method of Apostolidis et al. (2007). Briefly, ETSPI sample solution (100 µl at concentration of 1 mg/ml) and α-amylase (100 µl at concentration of 1 mg/ml) were mixed and incubated for 10 min at 25 °C. Subsequently, starch solution (100 µl at concentration of 1% (w/v)) was added to mixture and the incubation was continued for 10 min at 25 °C. Afterwards, dinitrosalicylic acid reagent (200 µl) was added and the resulting mixtures were heated at 95 °C for 5 min. The resulting mixtures were cooled to room temperature

and diluted with 3 ml of deionized water prior to the absorbance measurement at 540 nm. The α-amylase inhibitory percentage was calculated using the equation below:

$$\% \text{ amylase inhibitory} = \frac{(A_{pos} - A_{blank}) - (A_{sample} - A_{blank})}{(A_{pos} - A_{blank})} \times 100\%$$

(eq. 3)

where A_{pos} was referred as absorbance of α-amylase and starch without addition of sample while A_{blank} was absorbances of blank, A_{sample} was absorbance of mixture of ETSPI solution and α-amylase solution, A_{blank} was absorbance of sample blank.

In vitro Angiotensin Converting Enzyme (ACE) Inhibitory Assay of ETSPI

The angiotensin converting enzyme (ACE) inhibitory activity of ETSPI was performed according to the method by Cushman and Cheung (1971). The ETSPI solution (100 µl at concentration of 1 mg/ml) was vigorously mixed with ACE solution (100 µl at concentration of 50 mU/ml) and pre-incubated at 37 °C for 10 min. Consequently, histidine-histidine-leucine (HHL, 300 µl) was added and the mixtures were incubated for another 30 min. Subsequently, 1 ml of 1 M HCl was added in order to terminate the reaction. The hippuric acid formed was then extracted using the 1.6 ml of ethyl acetate (Fisher Scientific, Malaysia). The upper fraction was collected and dried by using the Vacuum Concentrator 5301 (Eppendorf, Germany) for 1 h. The pellet obtain was then solubilized with 2 ml of deionized water and the absorbance of resulting solution was determined at 228 nm using a UV-vis spectrophotometer (Spectamax M5, Molecular Devices, USA). The inhibitory percentage was expressed using the following equation:

$$\% \text{ ACE inhibitory} = \frac{(A_{pos} - A_{blank}) - (A_{sample} - A_{blank})}{(A_{pos} - A_{blank})} \times 100\%$$

(eq. 4)

where A_{pos} was referred as the absorbance of ACE and HHL without addition of sample, while A_{blank} was absorbance of the blank, A_{sample} was absorbance of the mixture of ETSPI solution, ACE and HHL solution.

In vitro pancreatic lipase inhibiting assay of ETSPI

The lipase inhibitory activity of ETSPI was evaluated using the method as described by Shimura et al. (1992). A mixture of ETSPI solution (100 µl at a concentration of 1 mg/ml), phosphate buffer (0.5 ml, Bendosen Laboratory Chemicals, Malaysia) and olive oil (100 µl, Bertolli®) were pre-incubated for 10 min at 37 °C. Subsequently, the lipase solution (100 µl at a concentration of 0.71 mg/ml) was added into the resulting solution prior to incubation for 1 h at 37 °C. The reaction mixtures were then terminated at 95 °C for 10 min. The resulting of fatty acid released upon the reaction mixtures was determined using the titration with 0.1 N NaOH. The lipase inhibitory percentage was calculated based on the equation below.

$$\% \text{ lipase inhibitory} = \frac{V_e - V_s}{V_s} \times 100$$

(eq. 5)

where V_e was referred as the titration volume for the control (containing lipase and olive oil without ETSPI) whereas V_s

was referred as the titration volume for the sample (containing lipase, olive oil and ETSPI).

Statistical Analysis

The statistical results were performed by using SPSS version 21 (IBM, USA). One-way ANOVA was carried out and the means of results were compared by using DUNCAN multiple-range tests ($p<0.05$). All the analyses were conducted in at least 3 replicates, and in each replicate, triplicates were used to obtain an average data.

RESULTS

Effect of pH, Sample to Buffer Ratio, Temperature and Time on Extraction Yield

As shown in Figure 1, the pH did not show a significant ($p>0.05$) change from pH 6 to 6.5. However, the yield was significantly ($p<0.05$) increased from pH 6.5 to 8, and the highest yield up to 21.06 mg/g was obtained. As for the effects of sample-to-buffer ratio and temperature, the trends of protein yield were significantly ($p<0.05$) increased when both of these parameters increased. On the other hand, the time showed significant ($p<0.05$) increase from 0.5 to 1 h. However, this parameter did not show any significant ($p>0.05$) changes between 1 to 1.5 h. The yield was then increased from 1.5 to 2 h, and reached the highest value of protein yield (14.26 mg/g). After 2 h of extraction, the protein yield decreased. Through these findings, it was found that pH 8, 1:50 w/v. 2 h, and 80 °C were considered as the suitable parameters in obtaining the highest yield of protein. The result showed that the crude protein yield obtained was 4.4% (w/w, dry basis), as shown in Table 1.

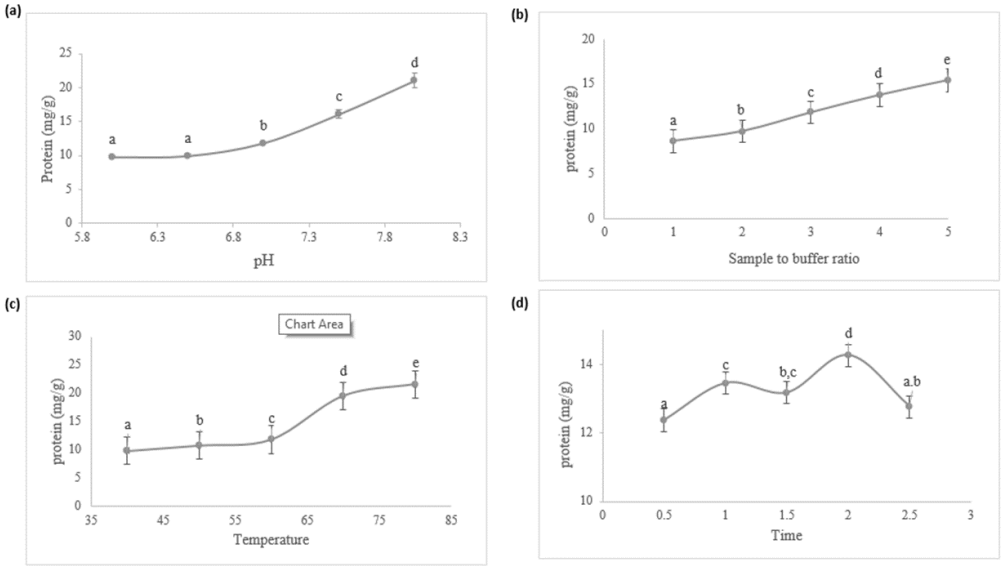

Figure 1. Effect of parameters (pH, sample to buffer ratio, temperature and time) towards the extraction yields

Table 1

Extraction yields, in vitro *digestibility, antioxidant activities and biological activities of ETSPI*

Extraction Yields and Activities	Values
Extraction yield, dry basis (%, w/w)	4.4 ± 0.03
In vitro digestibility activity (%)	72.5 ± 1.2
Antioxidant activities	
(a) ABTS (%/mg)	33.4 ± 3.3
(b) FRAP (mM $FeSO_4$/mg)	0.97 ± 0.02
ACE inhibitory activity (%/100µg)	56.6 ± 3.5
α -amylase inhibitory activity (%/100µg)	31.2 ± 1.8
Lipase inhibitory activity (%/100µg)	26.7±1.2

Data points are mean ± standard deviation (*n*=3)

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Based on Figure 2, the ETSPI profile has the molecular weight bands in a range of 13 to 54 kD. The major intense band appeared at 31 kD corresponding to 7S globulin (vicilin subunit), which is one of the major storage proteins in seeds. In addition, the subunit of legumin (11S globulin) was also discerned with 2 basic bands at 21-22 kD and one minor acidic band at 44 kD (Barac et al., 2010).

Fourier Transform Infrared (FTIR) Spectroscopy

Based on Figure 3, the FTIR spectra shows the prominent bands of protein in the region of 1654 cm^{-1}, 1543 cm^{-1} and 1454 cm^{-1}, which representing the functional groups of amide I, amide II and amide III, respectively. Other peaks at wavenumber of 520, 964, 1009, 1049, 2265, 2376, 2862, 2928, and 3445 cm^{-1}, were also found in other proteins (Chen et al., 2013; Gupta et al., 2010), which verified that extract was protein.

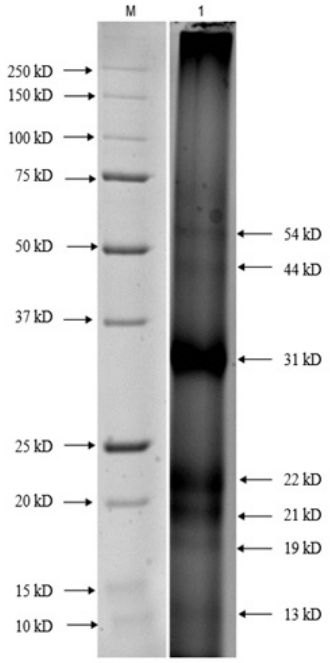

Figure 2. SDS PAGE profile of protein marker (Lane M) and ETSPI (Lane 1)

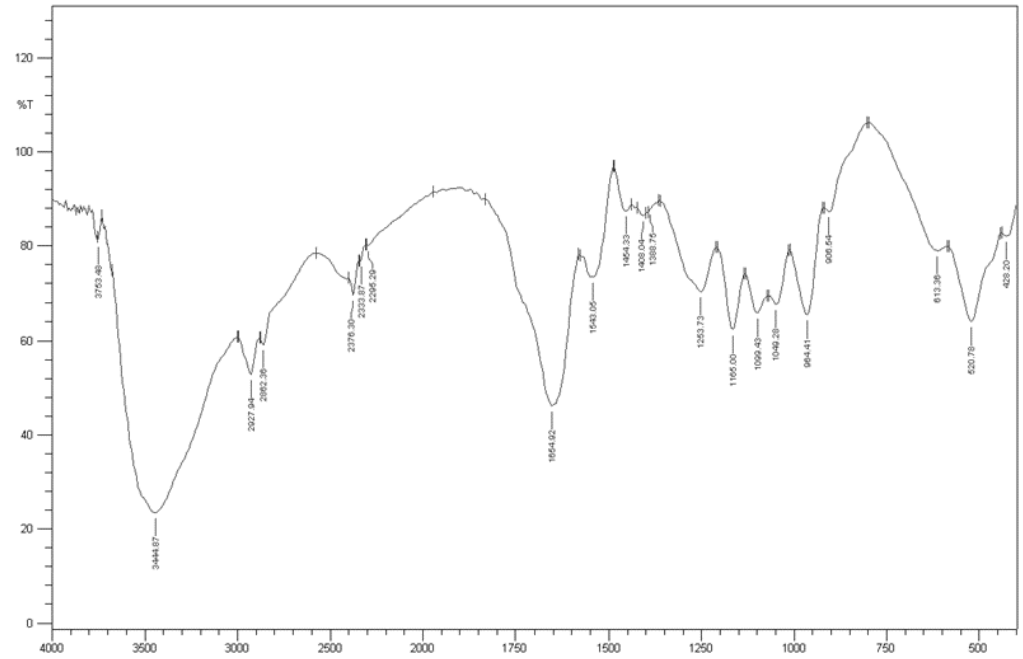

Figure 3. IR spectra of ETSPI

In vitro Digestibility

As shown in Table 1, ETSPI showed slightly lower the digestibility value (72.5%) as compared with Quinoa protein (78.4%) (Elsohaimy et al., 2015), cowpea protein isolate (78.7%) (El-jasser, 2010), and *Mucuna* bean protein (90.6%) (Adebowale et al., 2007).

Amino Acid Composition of ETSPI

The result showed that hydrophobic amino acids were dominant in ETSPI followed by acidic, uncharged polar and basic amino acids, as shown in Table 2. This characteristic amino acid profile was vital in correlation study of biological activities of ETSPI. Despite, the highest acidic amino residues such as aspartic and glutamic acid might suggest the protein was acidic in nature. Nonetheless, ETSPI was found lack in sulphur-containing amino acids, such as cystine and methionine. This could be revealed as the methionine was commonly limiting in legumes due to its low amount in storage protein, globulin (Lam & de Lumen, 2003). In fact, the aqueous-alkaline extraction used in isolating ETSPI might give an effect in term of the destruction of these amino acids.

Biological Activities of ETSPI

Table 1 shows that ETSPI has potential in inhibiting angiotensin converting enzyme (ACE) (56.6%), lipase (26.7%) and α-amylase (31.2%). Apart from that, the protein also exhibited ABTS free radical

Table 2

Amino acid composition of ETSPI based on their chemical characteristics

Characteristic of Amino Acid	Per 1000 Amino Residues	Percentage Based on Characteristic (%)
Acidic		
Asp	117	28.2
Glu	60	
Hydrophobic		
Ala	72	
Ile	43	
Gly	76	
Leu	70	38.0
Met	19	
Phe	35	
Pro	56	
Val	83	
Basic		
Arg	67	12.9
Lys	39	
Uncharged Polar		
Cys	9	
Thr	41	20.8
Tyr	21	
Ser	60	

Asp: Aspartic acid, Glu: Glutamic Acid, Ala: Alanine, Ile: Isoleucine, Gly: Glycine, Leu: Leucine, Met: Methionine, Phe: Phenylalanine, Pro: Proline, Val: Valiune, Arg: Arginine, Lys: Lysine, Cys: Cystine, Thr:Threonine, Tyr: Tyrosine, Ser: Serine

scavenging activity of 33.4% and ferric reducing antioxidant power (FRAP value) of 0.97 mM.

DISCUSSION

Based on a single factor experiment, it showed that all the parameters (i.e. pH, sample to buffer ratio, temperature and time) had shown the effects towards the extraction yield of protein. The incremental value of protein yield across the pH could be explained by the solubility and isoelectric point (*p*I) of protein. The solubility of protein commonly increases when the environmental pH of protein was above the *p*I value. As the phenomenon occurs, the net charge of the protein could be changed, resulting in the inducement in protein-solvent interaction (Samanta & Laskar, 2009). As a result, a better extraction yield of protein could be obtained at a higher pH condition. For the effect of sample-to- buffer ratio, the extraction yield increased as the ratio increased because higher amount of extraction buffer would encourage more proteins to be soluble. This might be due to the relaxation process of proton-transfer reaction between the buffer ions and

α-amino groups (Wijayanti et al., 2014). In other words, the increases of sample-buffer ratio will facilitate the extraction of the sample, thus increasing the protein yield. Temperature also plays an important role in the extraction procedure, as the increment of temperature will cause the protein become more porous, resulting in a higher efficiency of the protein to be solubilized in the buffer solution (Kumoro et al., 2010). For the effect of time, the longer extraction time was needed in the beginning phase of extraction as it could facilitate the solvent in diffusing the sample, which improved the protein yield. However, prolong extraction for a certain period of times could give adverse effect as the solubility of proteins could be decreased due to the formation of protein-phytate complexes (Eromosele et al., 2008). Hence, it could reduce the amount of protein yield. The pH 8, 1:50 (w/v), 80^{o}C and 1.5 h had shown the highest values of yield in the protein extraction within the studied parameters, and these parameters were used in extracting the ETSPI.

Sodium dedocyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted in determining the molecular weight of the protein through the electrophoretic mobility protein separation (Luo et al., 2004). Throughout the finding, ETSPI could be classified into the group 1 vicilin as its precursor polypeptide was fragmented in the range of 12-34 kD (Döring, 2015). Moreover, the trend of SDS-PAGE profile of ETSPI was similar to the protein profiles of pea seed, which the dominant protein was the 7S globulin subunit (Barać et al., 2015). Interestingly, this 7S globular protein was commonly delineated as an agent in inhibiting digestive enzymes, especially protease and amylase (Mundi, 2012). Despite, Lovati et al. (1998) had reported that the 7S globular protein from soybean protein could reduce the plasma cholesterol level up to 35%. This finding elucidated that the major protein fraction in this ETSPI could be part of the contributors towards its biological activities.

The FTIR analysis was performed in procuring the understanding of ETSPI protein structure. Amide I and amide II are most vital band of protein backbone as it could be used in prediction of secondary structure of proteins (Kong & Yu, 2007). From the result, it could be suggested that the secondary structure of ETSPI belongs to α-helical as the peak of amide 1 was located in the region of 1650 to 1660 cm^{-1} (Zhao et al., 2008).

Protein digestibility is one of the important factors in examining the nutritional quality of protein (Elsohaimy et al., 2015). The results had shown the digestibility was lower than other extract. This might attribute to the complex structure of protein such as globulin and high molecule weight of protein that present in the ETSPI, which might slow down the hydrolysis. In fact, the compact structure of protein might encumber the proteolytic enzyme in cleavage the peptide bond, and resulted a decline in digestibility.

Amino acid composition was conducted in studying its correlation with the biological activity. Throughout drug discovery research, the enzyme inhibitor was commonly worked

by interacting target enzyme through non-covalent force such as hydrophobic force, electrostatic force, hydrogen bond and Van der Waals forces. This conceptual was implemented in our research and it could be suggested that amino acid presence in ETSPI might interacted with our target enzyme (lipase, ACE, amylase) through interaction forces which resulting the inhibitory activity as shown on Table 1. Hernández-Ledesma et al. (2011) reported that ACE preferred to interact with inhibitor or substrate, which had the hydrophobic amino residue at N-terminal. Nonetheless, the bond of certain hydrophobic amino acid, such as Leu and Pro, to the active site of ACE through hydrogen and electrostatic interaction might cause the enzyme to lose its ability in binding to the substrate (Jang et al., 2011; Pan et al., 2011). In addition, Ashok and Aparna (2017) reported that bioactive peptide, which contain Val and Ala mimicked the interaction of lisinopril (ACE synthetic drug inhibitor), thus reduced the availability of the catalytic or substrate binding sites for the substrate. This similar interaction could occur when the ETSPI, which has a high abundance in Leu, Val, Ala were added to the ACE. Another possible explanation on the reduced activity was the ETSPI might also interact with the His-His-Leu (substrate), in which lowering the susceptibility of the substrate to be bound by ACE. Interestingly, ETSPI had manifest a favorable ACE inhibitory activity as compared with other seed protein extracts (e.g. flaxseed, chickpea and pea), which all of them were reported no ACE inhibitory activity (Barbana & Boye, 2010; Marambe et al., 2008). Therefore, it was suggested that ETSPI has the potential in antihypertensive effect. On the other notes, the relative high amount of Gly, Leu, Ala and Arg in ETSPI was one of the attributions of the α-amylase inhibitory activity. These similar residues were reported to be abundant in soybean and oat, which both of these legumes were potential antidiabetic agents (Lacroix & Li-Chan, 2012). In fact, Mojica and de Mejía (2016) reported that Leu in their peptide (Leu-Ser-Lys-Ser-Val-Leu) had shown the highest interaction with α-amylase active site through the hydrophobic interaction. This hydrophobic amino acid residue of Leu is important in α-amylase inhibition as it blocks the ion channel of enzyme and thus, reduces the enzyme activity (van der Veen et al., 2001). It was also suggested that ETSPI might interact with starch and prevented the formation of glycosyl-enzyme intermediate. However, ETSPI had manifest slightly low activity as compared to barley protein isolated (51-57.3%) (Alu'Datt et al., 2012). This might be related to the compact structure of ETSPI and entrapment of active functional amino acid in the core of the protein. However, the activity could be enhanced by releasing the bioactive peptide throughout the enzymatic hydrolysis of parental chain protein (Elias et al., 2008). Thus, it could suggest that ETSPI might be a precursor in producing the bioactive peptide to enhance the inhibitory activity of α-amylase. In terms of lipase inhibitory activity, Arg was reported to contribute to the activity in lowering the cholesterol that

could relate to the antiobesity property (Oda, 2006). Despite, a protein comprising amino acid residues that are predominant with Glu and Pro , also reported to have the potential in inhibiting the lipase (Upadhyay et al., 2006). Apart from that, peptide with amino acid sequence of Cys-Gly-Pro-His-Pro-Gly-Gln-Thr-Cys, which adequately in inhibiting the pancreatic lipase was selected from phage displayed (Lunder et al., 2005). These findings supported that the result of which these amino acids were found relatively high amount in ETSPI. The olive oil, which was used as the substrate, might form emulsion with ETSPI, where the protein chain of ETSPI wraps over the oil droplets and thus, prevented the lipase from approaching them. Additionally, the inhibitory activity of lipase enzyme of ETSPI slightly similar compare to the other lipase inhibitory activity of seed extract, such as *Trigonella foenum-graecum* (30%) *Bunium persicum* (28%), and *Nigella sative* (31%) (Birari & Bhutani, 2007). In this comparison, secondary metabolite seed extracts were used instead of protein because there were no reports on lipase inhibitor protein available. Throughout these findings and supportive literature, it could suggest that ETSPI might be nominated as a novel lipase inhibitor. The hydrophobic properties were also considered as an important factor for the accessibility of the antioxidant protein to the cellular target organ via hydrophobic interaction with the bilayer lipid membrane. In fact, the hydrophobic group of amino acids, such as Phe, Trp, Tyr and His, should be credential for the antioxidant activity due to their chemical structure and functional group. For instance, the presence of the imidazole ring in His could act as proton donor, which neutralize the free radicals of ABTS or DPPH (Samaranayaka & Li-Chan, 2011). On the other hands, the relative high amount of Glu and Asp in ETSPI might also attribute to the antioxidant activity. It was elucidated that these amino acids were considered as metal chelating agents, which had potential in binding metal ions (Wang et al., 2009). The tendency of these amino acids in donating their excess electron towards the free radical could be one of the antioxidative abilities of ETSPI (Udenigwe & Aluko, 2012).

CONCLUSION

In summary, it can be verified that ETSPI present in the local medical drink had partially contributed to the therapeutic effects, such as antidiabetic, antihypertensive, antiobesity as well as antioxidant activities. The best extraction yield of ETSPI was obtained under the condition of extraction time of 2 h, buffer to sample ratio of 1:50 (w/v), temperature at 80 ^{o}C, and pH 8. In fact, the requisite amount of amino acids in ETSPI was the vital criterion due to its correlation with the biological activities. However, ETSPI had manifested mild activity in terms of biological activities due to the tertiary structure of the polypeptide which cause inaccessible of amino acid to the target enzyme or prooxidant. This limitation could be overcome by disrupting the tertiary structure through enzymatic hydrolysis of the isolated protein to bioactive peptide,

and this approach is suggested in our future research which will be focusing on identification of bioactive peptide.

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Antioxidant and Antidiabetic Effects of *Garcinia schomburgkiana* Extracts and Fermented Juices

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ABSTRACT

This study investigated total phenolic contents, antioxidant activities, and antidiabetic activities in flesh, seed and leaf extracts, and the fermented juices of *Garcinia schomburgkiana* Pierre (Madan). Seed extract showed the highest total phenolic content, radical scavenging activity and amylase inhibitory activity, whereas the leaf extract had the strongest ferrous ion chelating activity and inhibitory activities on glucosidase and lipase. The very strong positive correlation between total phenolic content and radical scavenging activity, and between ferrous ion chelating and glucosidase inhibitory activities were significantly found (r = 1.000 and 0.998 at $P<0.05$, respectively). Moreover, the Madan juice revealed the highest total phenolic content at 72 hour fermentation. However, production of radical scavenging and ferrous ion chelating activities of fermented juices increased at the start of fermentation. Additionally, amylase inhibitory activity increased during 24 hour fermentation, whereas glucosidase and lipase inhibitory activity reached maximal levels during 17 day and 48 hour fermentation, respectively. The total phenolic contents of the fermented juices were very strongly negatively correlated with radical scavenging activity, and ferrous ion chelating activity, significantly (r = -0.869 and -0.937 at $P<0.05$, respectively). In addition, the very strong positive correlation between radical scavenging activity and ferrous ion chelating activity was significantly found (r = 0.804, $P<0.05$). Our results indicated that *G. schomburgkiana* Pierre is a potential nutraceutical source, and its fermented juice can be further improved as a healthy fruit drink.

Keywords: Antidiabetic activity, antioxidant activity, *Garcinia schomburgkiana* Pierre

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INTRODUCTION

Obesity is a major non-communicable disease (NCD) which is becoming a worldwide concern. It is also a cause of other NCDs (i.e. cardiovascular risk factors, hypertension, and hyperinsulinemia including type II diabetes mellitus) and death. In Thailand, the prevalence of obesity shows an increasing trend, with approximately 33% and 43% of males and females affected, respectively (Teerawattananon & Luz, 2017). Moreover, the age-adjusted prevalence of people with diabetes in Thailand increased from 7.7% in 2004 to 9.9% in 2014 (Aekplakorn et al., 2018). Nowadays, one of the most common NCDs associated with obesity is type II diabetes mellitus, which is a metabolic dysfunction characterized by insulin resistance**,** insufficient insulin, inappropriate glucagon secretion, hyperglycemia, and glucotoxicity. Type II diabetes mellitus is a critical problem of global public health (Janghorbani et al., 2013), especially in developing countries. In a recent report, it has been predicted that there will be a further increasing trend of 382 million people with diabetes in 2013 rising to 592 million in 2035 (Guariguata et al., 2014). Meanwhile, several therapeutic methods have been developed to decrease hyperglycemia in Thai patients with type II diabetes mellitus. Many reports have focused on key enzymes in type II diabetes mellitus such as lipase, α-glucosidase, and α-amylase. The α-glucosidase and α-amylase enzymes showed functions involving polysaccharide catabolism and starch hydrolysis which lead to a lower increased blood glucose level after a mixed carbohydrate diet (Kwon et al., 2006). For the lipase enzyme, it shows the effect of lowering the increased plasma triglyceride levels after high fat diets (Zhang et al., 2008). Moreover, the presence of bioactive compounds found in plants (i.e. phenolic agents) may be of medicinal significance (i.e. antioxidant activities). Antioxidants are a major inhibitor in glycoside hydrolase activities, glycation reaction, and production of advanced glycation end-products (AGEs) (Adefegha et al., 2015). Previous study has been revealed that correlation between total phenolic content, antioxidant activity, and amylase inhibitory activity of bioprocessed local fruit extracts (pineapple and guava) are investigated to improve the value of fruit wastes (Sousa & Correia, 2012).

Finding novel antidiabetic drugs from plant extracts with fewer side effects, therefore, may be useful for human health worldwide. *Garcinia schomburgkiana* Pierre is a local fruit tree known as Madan that is commonly found in the central and southern regions of Thailand, especially in Nakhon Nayok province. Its leaves and fruit can be consumed either raw or cooked, but this is of limited popularity due to its sourness (Subhadrabandhu et al., 2001), and its seeds are usually discarded as waste during fruit processing. However, *G. schomburgkiana* Pierre has been reported to have high phytochemical content, high total phenolic content and high antioxidant property in root and branch acetone extract (Meechai et al., 2016a, 2016b, 2016c). Promoting consumption of the processed fruit could be an excellent way to improve the economic viability of Madan, with products such as healthy fruit drink and fruit compote.

However, the health effect of processing on the activities of lipase, amylase, and glycosidase, including antioxidant capacities of this fruit, are little known and poorly understood. Therefore, our major goals focused on the effect of fermented juice and extracts from *G. schomburgkiana* Pierre as an antioxidant and antidiabetic drug. This work was carried out in Ongkarak district, Nakorn Nayok province.

MATERIALS AND METHODS

Chemicals

Orlistat (Sigma), triolein (Sigma-Aldrich), sodium dihydrogen phosphate dehydrate (Sigma-Aldrich), ethanol absolute (Merck), Di-potassium hydrogen phosphate anhydrous (Merck), 4-nitrophenyl-alpha-D-glucopyranoside (PNPG) (Sigma), 3,5-dinitrosalicylic acid (Sigma), DTNB (5,5′-dithiobis, 2-nitrobenzoic acid) (Sigma), ethanol solution (Sigma), Tween 40 (Merck), α-amylase from porcine pancreas (Sigma-Aldrich), α-glucosidase from *Saccharomyces cerevisiae* (Sigma-Aldrich), lipase from *Candida Antarctica* (Sigma-Aldrich), reduced glutathione solution (GSH) (Sigma), acarbose (Sigma), sodium potassium tartrate (Merck), phenolphthalein indicator (Merck).

Sample Collection

Leaves and fruits of *G. schomburgkiana* Pierre were collected from Ongkarak district, Nakorn Nayok province, then washed with distilled water and kept in a plastic bag at 4°C until used.

Aqueous Fermented Extracts

Fresh fruit samples of *G. schomburgkiana* Pierre without seeds were cut into small pieces. After that, they were mixed with distilled water at ratios of 1:1, 1:2, 1:3, and 1:4. The mixtures were combined with brown sugar 2% (w/v), and then left at room temperature to ferment. Each fermented juice was collected in 50 ml polyethylene tubes at 0 hours, 24 hours, 48 hours, 72 hours, 10 days, 17 days, and 24 days, and kept at -20°C until used.

Plant Extracts

Seed, flesh and leaf samples of *G. schomburgkiana* Pierre were cut into small pieces, incubated at 50°C for about 24 hours and then milled using a homogenizer. After that, 50 g of the powder was added by 95% ethanol and mixed, then incubated at 37°C for 14 hours with shaking. The extracts were filtered through cheesecloth, and all filtrates were evaporated at 45°C for 2 hours by vacuum evaporator (IKA[a] RV10), weighed and adjusted the volume with 95% ethanol solvent to the final concentration of 1 g/ml. This was then diluted to 1:1000, 1:100, 1:50, and 1:10 with 95% ethanol solvent, and kept at -20°C until used. Each sample was extracted in duplicate (Thummajitasakul et al. 2014; Thummajitsakul & Silprasit, 2017).

Total Phenolic Contents

Total phenolic contents were determined with the Folin–Ciocalteu colorimetric technique. Each extract (300 µl) was

combined with 1.5 ml of Folin-Ciocalteu reagent and left for 5 min. After that, 1.2 ml of sodium carbonate (7.5% w/v) was added and incubated for 30 min at room temperature. The absorbance of each reaction was then measured at wavelength 765 nm by a spectrophotometer (Model T60UV). Each assay was carried out in duplicate. Gallic acid was used to generate a calibration curve ($y=5.32x-0.02$; $R^2=0.96$) (Deetae et al., 2012). Total phenolic contents were then shown in unit of mg gallic acid equivalent per gram extract.

ABTS Method

ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium] radical cation was prepared from 7 mM ABTS solution (10 ml) and 140 mM potassium persulfate (179 µl), then incubated for 12-16 hours in the dark at room temperature. After that, the ABTS radical cation was diluted with distilled water until its absorbance reached 0.700 ± 0.050 at 734 nm. Then 20 µl of each extract was reacted with 3.9 ml of the diluted ABTS radical solution for 6 min in the dark at room temperature, and the absorbance was quickly measured at 734 nm. Each assay was performed in duplicate. The antioxidant capacity was calculated using the below formula (Deetae et al., 2012).

$$\% \text{ Antioxidant capacity} = \frac{(A_{ABTS} - A_{sample}}{A_{ABTS}} \times 100 \quad (1)$$

where A_{ABTS} was the absorbance of the $ABTS^{\bullet+}$ solution without a sample, and A_{sample} was the absorbance of the $ABTS^{\bullet+}$ solution with a sample.

FIC Method

The ferrous ion chelating activity was measured by mixing each sample extract (1 ml) with 0.1 mM $FeSO_4$ (1 ml) and 0.25 mM ferrozine (1 ml). This was left at room temperature for 10 min in the dark, following which the absorbance of each reaction was measured at 562 nm. EDTA was used as a standard solution. Each reaction was performed in duplicate. The percentage of metal chelating ability was calculated according to the below formula (Deetae et al., 2012).

$$\% \text{ Metal chelating capacity} = 1 - \frac{(A_{sample} - A_{sample\ blank})}{(A_{water} - A_{water\ blank})} \times 100 \quad (2)$$

where A_{sample} and $A_{sample\ blank}$ were the absorbance of a sample with ferrozine and without ferrozine, respectively.

A_{water} and $A_{water\ blank}$ were the absorbance of distilled water with ferrozine, and without ferrozine, respectively.

Lipase Inhibitory Activity

Lipase inhibitory activity was determined by mixing each extract (900 µl), 1% triolein in Tween 40 (1 ml), and 50 mM sodium phosphate buffer pH 8.0 (400 µl). This was then incubated at 37°C for 30 min. After that, 0.15 unit/ml lipase solution (400 µl) was gently mixed, before incubation at

37°C for 1 hour. For the blank solution, sodium phosphate buffer was used in place of enzyme solution, then each reaction was mixed with 95% ethanol (1 ml), and titrated with 0.025 N NaOH until the phenolphthalein indicator changed to pink (Jagdish et al., 2013; Wrolstad et al., 2005). Orlistat was used as a positive control. Each extract was performed in duplicate. The percentage of lipase inhibition was calculated using the below formula.

$$\textbf{\%}\ \text{Lipase inhibition} = \frac{[(A-a)-(B-b)]}{(A-a)} \times 100 \quad (3)$$

where A and B were the volume of NaOH used until the end-point was reached, for titration of distilled water and extract with lipase, respectively.

a and b were the volume of NaOH used until the end-point was reached, for titration of distilled water and extract without lipase, respectively.

Amylase Inhibitory Activity

Amylase inhibitory activity was measured by mixing between 12 units/ml amylase solution (100 µl) with each extract (100 µl) and incubating at 25°C for 30 min. Then, 100 µl of 1% starch solution was added, and left at 25°C for 3 min. After that, 100 µl of DNS reagents (96 mM 3, 5-dinitrosalicylic acid and 5.3 M sodium potassium tartrate in 2 M sodium hydroxide) were added and incubated at 85°C for 15 min, then immediately cooled at 4°C. Additionally, distilled water (900 µl) was added and mixed, and the absorbance was detected at 540 nm (Wang et al., 2018). Acarbose was used as a positive control. Each extract was carried out in duplicate. The percentage of amylase inhibition was calculated using the following formula.

$$\textbf{\%}\ \text{Amylase inhibition} = 1 - \frac{(A_{sample} - A_{sample\ blank})}{(A_{water} - A_{water\ blank})} \times 100 \quad (4)$$

where A_{water} and A_{sample} were the absorbance of distilled water and sample extract with amylase, respectively.

$A_{water\ blank}$ and $A_{sample\ blank}$ were the absorbance of distilled water and sample extract without amylase, respectively.

Glucosidase Inhibitory Activity

Glucosidase inhibitory activity was determined in a reaction of 3 mM glutathione (25 µl), 67 mM potassium phosphate buffer pH 6.8 (250 µl), 0.3 unit/ml glucosidase (25 µl), and each extract (100 µl) at 37°C for 10 min. After that, 10 mM PNPG (25 µl) was mixed and incubated at 37°C for 10 min, then 0.1 M sodium carbonate (400 µl) was added with gently shaking. The absorbance was measured at 400 nm (Elya et al., 2012). Acarbose was used as positive control. The percentage of glucosidase inhibition was calculated following formula.

$$\%\ \text{Glucosidase inhibition} = 1 - \frac{(A_{sample} - A_{sample\ blank})}{(A_{water} - A_{water\ blank})} \times 100 \quad (5)$$

where A_{water} and A_{sample} were the absorbance of distilled water and sample extract with glucosidase, respectively.

$A_{water\ blank}$ and $A_{sample\ blank}$ were the absorbance of distilled water and sample extract without glucosidase, respectively.

Data Analysis

All tests were analyzed in duplicate, at minimum. Data were expressed as mean ±SD, and significant differentiation between mean values was evaluated by one-way variance analysis (ANOVA). The principal component analysis (PCA) with varimax rotation was used to determine correlations among variables. Linear regression analysis (R^2 = 0.74-1.00) was applied to estimate EC50 values (effective concentration of each extract needed to scavenge ABTS radicals, chelate ferrous ions, or inhibit enzymes by 50%), which were consequently adjusted to 1/EC50 (Thummajitsakul et al., 2014). All statistics were calculated via Paleontological statistic program version 3.16 (Hammer et al., 2001) and PSPP program version 0.10.5 (Pfaff et al., 2013).

RESULTS AND DISCUSSION

Nowadays it is well known that amylase and glucosidase are enzymes for carbohydrate digestion in the digestive system. Amylase is responsible for breaking down 1, 4-glycosidic bonds of polysaccharides to disaccharides, whereas glucosidase digests the disaccharides to monosaccharides, which leads to an increase of the postprandial plasma glucose. Therefore, inhibition of these enzyme activities can help to delay carbohydrate digestion or extend time in digestion, which causes a reduction in glucose absorption and postprandial hyperglycemia (Yilmazer-Musa et al., 2012). In addition, lipase is an enzyme responsible for fat digestion and absorption, which consequently leads to hyperlipidemia (Kershaw et al., 2006). Therefore, lipase is also a key enzyme in preventing obesity and obesity-related diseases, inhibition of which can help to delay lipid absorption (Padwal & Majumdar, 2007).

In our study, the results demonstrated that total phenolic content, radical scavenging activity, ferrous ion chelating activity, and antidiabetic potential were found in 95% ethanol extracts of seed, flesh, leaf, and fermented juices from *G. schomburgkiana* Pierre. Total phenolic contents of seed, leaf, and flesh extracts were 110.03±21.60, 60.89±8.55, 6.21±2.85 mg gallic acid/g extract, respectively. The EC50 values of each extract and fermented juice for radical scavenging, ferrous ion chelating, and enzyme inhibitory activities were adjusted to 1/EC50. The higher 1/EC50 values indicated higher biological activities. The corresponding order of the 1/EC50 of radical scavenging activities was 0.1335, 0.0724, and 0.0011 for seed, leaf, and flesh extracts, respectively. However, ferrous ion chelating activity showed the highest 1/EC50 value in leaf extract (0.5128), followed by seed extract (0.0525), and flesh extract (0.0287). Additionally, amylase inhibitory activities were observed in seed, flesh and leaf extracts that showed 1/EC50 values in order 0.0277, 0.0192 and

0.0186, whereas the order of glucosidase and lipase inhibitory activity was leaf extract (0.0801 and 0.0450), seed extract (0.0675 and 0.0309), and flesh extract (0.0658 and 0.0208) (Table 1).

Table 1
Total phenolic contents, radical scavenging activities, ferrous ion chelating activities, inhibitory activities on amylase, glucosidase and lipase of 95% ethanol extracts of seed, flesh and leaf from G. schomburgkiana *Pierre*

Biological activities	95% ethanol extracts		
	Seed	Flesh	Leaf
Total phenolic contents (mg gallic acid/ g extract)	110.03±21.60	6.21±2.85	60.89±8.55
Radical scavenging activities EC50 (1/EC50)	7.49±0.92 (0.1335)	859.86±45.72 (0.0011)	13.81±1.91 (0.0724)
ferrous ion chelating activities EC50 (1/EC50)	19.03±11.62 (0.0525)	34.85±24.70 (0.0287)	1.95±0.85 (0.5128)
Amylase inhibition EC50 (1/EC50)	36.15±1.72 (0.0277)	52.06±0.01 (0.0192)	53.60±2.03 (0.0186)
Glucosidase inhibition EC50 (1/EC50)	14.81±6.19(0.0675)	15.19±4.85 (0.0658)	12.48±0.21 (0.0801)
Lipase inhibition EC50 (1/EC50)	32.33±7.95 (0.0309)	48.09±1.67 (0.0208)	22.21±8.36 (0.0450)

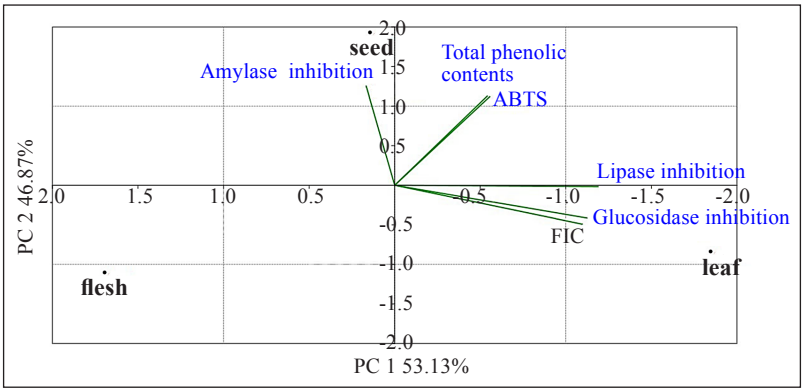
A recent study of ethanolic extract of *Garcinia mangostana* Linn., which revealed hypoglycaemic activity by the increase of the number of insulin-producing β-cells in rat (Taher et al., 2016), support our result. Generally, appropriate reactive oxygen species (ROS) are useful for many biological processes in the human body such as immune function, gene expression and cellular responses. However, an excess ROS can attack insulin-producing pancreatic β-cells, which is one of the major causes of type II diabetes mellitus (Tangvarasittichai, 2015). Moreover, obesity can trigger an increase in ROS, which is linked to the development of obesity-related disease (Marseglia et al., 2015).

In addition, our result indicated the moderate level of ABTS radical scavenging activities and total phenolic contents in ethanolic extract from *G. schomburgkiana* leaf. Similarly, previous study shows low total phenolic contents (58.84, 37.68, and 97.97 mg of gallic acid/g extract), and moderate ABTS radical scavenging activities (151.51, 31.21, and 93.19 mg of g extract) have been found in dichloromethane, acetone, and methanol extracts of *G. schomburgkiana* leaf, respectively (Meechai et al., 2016a). However, acetone extracts of roots and branches of this plant show high phenolic contents, antioxidant capacity, and radical scavenging activity (Meechai et al., 2016a, 2016b).

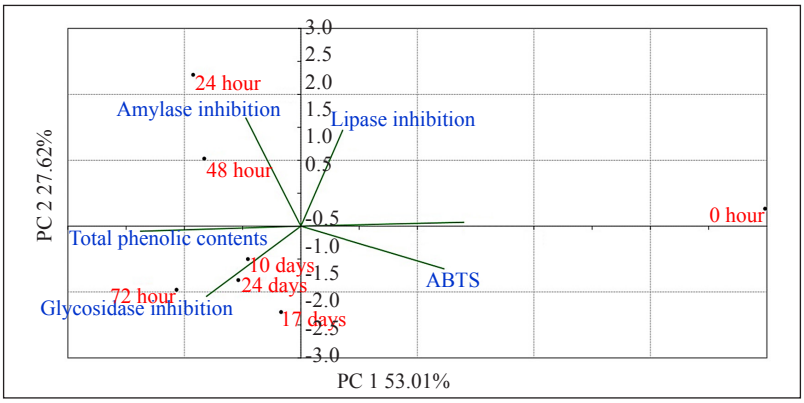
Moreover, parts of *G. schomburgkiana,* namely bark, branch, root, wood, and fruit have been reported as natural sources of phenolics such as xantones, flavonoid, biphenyls, depsidones, biflavonoids, and benzophenones (Ito et al., 2013; Le et al., 2016; Meechai et al., 2016c; Mungmee et al., 2013; Sukandar et al., 2016; Vo et al., 2012). These phenolics reveal many biological capacities such as antidiabetic and antiobesity activities (Liu et al., 2015; Trinh et al., 2017; Vinayagam & Xu, 2015). Therefore, several extracts from plant sources are used as a phenolic reservoir in effective interaction or inhibition on the key enzymes which are linked to type II diabetes and obesity. Interestingly, seed extract showed lower ferrous ion chelating activities, glucosidase inhibitory activity, and lipase inhibitory activity in comparison to leaf extracts, whereas total phenolic content, radical scavenging activity, and amylase inhibitory activity were higher. Based on previous studies, seeds of several fruits consist of abundant phenolic contents and antioxidant activities (Babbar et al., 2011).

The PCA results of the ethanolic extracts revealed that total phenolic content, radical scavenging activity, ferrous ion chelating activity, and inhibitory activities on amylase, glucosidase, and lipase were grouped into 2 components with eigenvalues above 1. PC1 and PC2 accounted for 53.13% and 46.87% of the total variance, respectively. PC1 contained total phenolic content, radical scavenging activity, and amylase inhibitory activity, whereas PC2 contained ferrous ion chelating activity, glucosidase inhibitory activity, and lipase inhibitory activity. It confirmed that the highest value of total phenolic content, radical scavenging activity, and amylase inhibitory activity were found in the seed extract, whereas the highest value of ferrous ion chelating activity, glucosidase inhibitory activity, and lipase inhibitory activity were found in the leaf extract (Figure 1A). Furthermore, the results showed that total phenolic content was very strongly correlated with radical scavenging activity ($r = 1.000$, $P < 0.05$), and ferrous ion chelating activity was very strongly correlated with glucosidase inhibitory activity, significantly ($r = 0.998$, $P < 0.05$).

It is possible that the antioxidant activities found in all extracts are contributed by the phenolic agents found in this plant, and that the glucosidase inhibition activities are provided by phytochemical constituents which exhibit ferrous ion chelating activities. It has been reported that dichloromethane, acetone, and methanol extracts of *G. schomburgkiana* contain many biological agents, namely benzoic acid, vanillin, citric acid, linoleic acid, oleic acid, protocatechuic acid, catechol, and phloroglucinol (Meechai et al., 2016c). Moreover, GC-MS analysis of phytochemical compounds in acetone extracts of *G. schomburgkiana* branch shows that antioxidant activity is contributed by phenolic compounds (isovanillic acid and 2,6-dihydroxy-4-methoxybenzophenone) and fatty acids in the extract (Meechai et al., 2016a).



(A)



(B)

Figure 1. Principal component analysis (PCA). A is PCA of seed, flesh and leaf extracts of *G. schomburgkiana* Pierre. B is PCA of fermented juices of *G. schomburgkiana* Pierre. ABTS was expressed as radical scavenging activities, and FIC was expressed as ferrous ion chelating activities

In addition, it has been reported that several phenolic compounds (i.e. methyl 4-O-galloylchlorogenate, 4-O-galloylchlorogenic acid, methyl chlorogenate, dihydromyricetin, quercitrin, myricitrin, catechin, epicatechin, gallocatechin, and gallic acid) from plants have health-related benefits, and exhibit antioxidant properties (Parik & Patel, 2017) including inhibitory activities on amylase, glucosidase, and lipase (Kamiyama et al., 2010; Li et al., 2009; McDougall et al., 2005; Yang et al., 2014; Yilmazer-Musa et al., 2012), which have key roles in the control of diabetic and obesity-related diseases in humans.

Moreover, our results also indicated that total phenolic content, antioxidant activity and antidiabetic activity were found in the fermented juice of *G. schomburgkiana* Pierre. Total phenolic contents of the fermented juices increased to a maximum at 1.06±0.36 mg gallic/g fresh fruit after 72 hour fermentation, and then gradually decreased. However, the radical scavenging activity and ferrous ion chelating activity of the

fermented juice showed the highest 1/EC50 value at the start of fermentation (1/EC50 = 0.67 x 10^{-3} and 4.83 x 10^{-3}, respectively), and then they again increased to 0.47 x 10^{-3} and 0.80 x 10^{-3} on 17 and 24 days after the start of fermentation, respectively (Table 2). The results of amylase inhibitory activity showed the highest 1/EC50 level (11.38 x 10^{-3}) at 24 hour fermentation and then decreased. Glucosidase inhibitory activity in the fermented juice had maximal levels at 4.90 × 10^{-3} at 17 day fermentation and then reduced, and lipase inhibitory activity had the highest value (1/EC50 = 2.36 x10^{-3}) at 48 hour fermentation and then declined (Table 3).

The results from the fermentation process may involve in several mechanisms. Fermentation is a method of food preservation by microorganisms or enzymes based on converting carbohydrates to organic acids or alcohol, producing health-promoting bioactive agents, improving stability and functions of the bioactive

Table 2
Total phenolic contents, radical scavenging activities, and ferrous ion chelating activities of fermented juice from flesh of G. schomburgkiana *Pierre*

Fermentation time	Total phenolic contents (mg gallic/ g fresh fruits)	Radical scavenging activities		Ferrous ion chelating activities	
		EC50(mg fresh weight/ml)	1/EC50	EC50 (mg fresh weight/ml)	1/EC50
0 hour	0.33±0.07	1,501.36± 735.37	0.67 x 10^{-3}	207.13± 6.51	4.83 x 10^{-3}
24 hour	0.91±0.38	4,183.49± 636.99	0.24 x 10^{-3}	5,259.15± 530.19	0.19 x 10^{-3}
48 hour	1.02±0.34	5,595.61± 840.83	0.18 x 10^{-3}	1,628.77± 185.62	0.61 x 10^{-3}
72 hour	1.06±0.36	2,991.89± 364.10	0.33 x 10^{-3}	2,648.73± 830.94	0.38 x 10^{-3}
10 days	0.96±0.44	2,581.04± 637.19	0.39 x 10^{-3}	2,002.68± 85.03	0.50 x 10^{-3}
17 days	0.80±0.29	2,140.76± 532.54	0.47 x 10^{-3}	1,877.75± 330.11	0.53 x 10^{-3}
24 days	0.92±0.31	3,788.18± 488.48	0.26 x 10^{-3}	1,245.41± 240.40	0.80 x 10^{-3}

Table 3
Inhibitory activities on amylase, glucosidase and lipase by fermented juices from flesh of G. schomburgkiana *Pierre*

Fermentation time	Amylase inhibition		Glucosidase inhibition		Lipase inhibition	
	EC50 (mg fresh weight/ml)	1/EC50	EC50 (mg fresh weight/ml)	1/EC50	EC50 (mg fresh weight/ml)	1/EC50
0 hour	648.77± 87.46	1.54 x 10^{-3}	121,077	8.26 x 10^{-6}	479.79±11.90	2.08 x 10^{-3}
24 hour	87.89± 32.35	11.38 x 10^{-3}	587.47±17.74	1.70 x 10^{-3}	436.73±18.06	2.29 x 10^{-3}
48 hour	256.84± 123.69	3.89 x 10^{-3}	535.61±24.74	1.87 x 10^{-3}	424.17±27.56	2.36 x 10^{-3}
72 hour	559.02± 118.82	1.79 x 10^{-3}	249.92±144.91	4.00 x 10^{-3}	807.76±12.62	1.24 x 10^{-3}
10 days	219.52± 151.16	4.55 x 10^{-3}	603.18±9.64	1.66 x 10^{-3}	2142.12±449.22	4.67 x 10^{-4}
17 days	642.34± 48.97	1.56 x 10^{-3}	204.24±52.97	4.90 x 10^{-3}	711.90±76.97	1.40 x 10^{-3}
24 days	583.20± 23.21	1.71 x 10^{-3}	513.20±13.58	1.95 x 10^{-3}	1803.33±440.23	5.55 x 10^{-4}

agents (Tamang et al., 2009, 2016; Thapa & Tamang, 2015). Further to this, it has been reported that several isolates of natural lactic acid bacteria (i.e. *Lactobacillus brevis*, *Lactobacillus pentosus* and *Lactococcus lactis*) were found in pickled *G. schomburgkiana* Pierre (Madan) from local markets at Nakhon Nayok province and Nakhon Ratchasima province in Thailand (Chanprasert & Gasaluck, 2011). However, phenolic levels can be reduced in fermented juices by oxidation or polymerization with biomolecules (i.e. phytochemicals carbohydrates, lipids, and proteins) that induce precipitation of phenolic and biomolecules during fermentation (Chen et al., 2018; Manach et al., 2004; Parada & Aguilera, 2007; Zou et al., 2017). In contrast, radical scavenging activity, ferrous ion chelating activity, inhibitory activities on amylase, glucosidase and lipase of the fermented juices were higher during fermentation. These results can be explained by the reaction of enzymes and organic acid from bacteria or fruits which may help to release phenolics or antioxidants from complicated forms in fruit fiber into free forms. Similar evidence supports our results that total flavonoid content in papaya juice increases during fermentation, but phenolic compounds have the highest level at 30 hours and then decrease (Chen et al., 2018).

The PCA result of the fermented juice showed that total phenolic content, radical scavenging activity, ferrous ion chelating activity, and inhibitory activities on amylase, glucosidase and lipase were also divided into 2 components. PC1 and PC2 accounted for 53.01% and 27.62% of the total variance, respectively. PC1 contained total phenolic content, radical scavenging activity, and ferrous ion chelating activity, whereas PC2 contained amylase inhibitory activity, glucosidase inhibitory activity, and lipase inhibitory activity. The result confirmed that total phenolic content and glucosidase inhibitory activity were at the highest levels in the fermented juices after fermentation for 72 hours and 17 days respectively, whereas radical scavenging activity and ferrous ion chelating activity showed the highest level at 0 hour, including amylase and lipase inhibitory activities which had the highest value at 24 and 48 hour fermentation, respectively (Figure 1B). Furthermore, total phenolic content was, significantly, very strongly negatively correlated with radical scavenging activity ($r = - 0.869$, $P < 0.05$) and ferrous ion chelating activity ($r = -0.937$, $P < 0.05$). In addition, radical scavenging activity was, significantly, very strongly positively correlated with ferrous ion chelating activity ($r= 0.804$, $P < 0.05$). It is possible that the presence of nonphenolic compounds in bioprocessed fruit may contribute to antioxidant activity, and hydrolysis of fruit by microorganism enzyme can produce complex mixture and bioactive agents that can disturb in antioxidant activity (Sousa & Correia, 2012). Moreover, the nonphenolic compounds (such as beneficial fatty acids) in acetone extracts of *G. schomburgkiana* branch and root may act as antioxidants (Meechai et al., 2016a).

Fermented fruits are functional foods with health-promoting properties for humans. Previously, several studies have reported that the presence of functional microorganisms in fermented food can promote human health by preventing non-communicable diseases such as cardiovascular disease, cancer, and diabetes (Swain et al., 2014). Overall, our study indicated that all ethanol extracts and fermented juices of *G. schomburgkiana* Pierre have interesting biological activities. The local fruit waste has also benefits as a natural source of bioactive agents such as phenolics, antioxidants, and antidiabetic agents. It also implies, however, that other phytochemical groups (i.e. alkaloids, terpenoids and hydroxycitric acid) in leaf extracts may help ferrous ion chelating activity and inhibitory activities of glucosidase and lipase. Earlier studies demonstrated that *Garcinia* sp. (i.e. *G. cambogia*, *G. atroviridis*, and *G. indica*) contains organic acid such as hydroxycitric acid, which displays anti-obesity activities (Chuah et al., 2013). Our studies, therefore, demonstrated for the first time that seeds generally discarded as waste had rich total phenolic content, radical scavenging activity, ferrous ion chelating activity, and antidiabetic potential, and that a distribution of the biological activities in different parts of *G. schomburgkiana* Pierre fruit was found. In addition, the nutritional values of this fruit can be improved by fermentation, allowing for further development as a healthy fruit drink.

CONCLUSION

We conclude that the total phenolic content, ferrous ion chelating activity, and radical scavenging activity including antidiabetic properties were found in seed, flesh, and leaf extracts, as well as the fermented juices of *G. schomburgkiana* Pierre. Seed extract showed the highest value of total phenolic content, radical scavenging activity, and amylase inhibitory activity, whereas the leaf extracts revealed the strongest ferrous ion chelating activity, and inhibitory activities on glucosidase and lipase. Furthermore, fermentation of *G. schomburgkiana* Pierre can provide nutraceutical capacities, namely radical scavenging activity, ferrous ion chelating activity, and inhibitory activities on amylase, glucosidase and lipase, which varied according fermentation periods. The extracts and fermented juices from *G. schomburgkiana* Pierre could, therefore, be developed as a tool to manage and prevent diabetic and obesity diseases (along with an appropriate healthy diet and physical activity), and the phytochemicals with antioxidant activities and antidiabetic activities in seeds of this plant should be studied further, including the efficacy of fermentation.

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The Monitoring of Organophosphorus and Carbamate Insecticides and Heavy Metal Contents in Paddy Field Soils, Water and Rice (*Oryza sativa* L.)

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ABSTRACT

The aim of this work was to monitor organophosphorus and carbamate insecticides in paddy field soils, water and rice plants by measuring anti-acetylcholinesterase activity, and heavy metal contents by an atomic absorption spectrometry. The results showed that the percentages of anti-acetylcholinesterase activities were found in the order of shoot > soil > root > grain > water. Concentrations of heavy metals in all samples were found in the following order: Fe>Mn>Ni>Pb>Zn, particularly Fe, Ni and Pb were found in concentrations exceeding their maximum permissible levels in all samples of water and rice plants. Interestingly, principal component analysis confirmed positive correlations (significantly: P <0.05) between the percentages of anti-acetylcholinesterase activities and heavy metal contents, between Zn and Mn contents, between Zn and Pb contents, between Pb and Mn contents, and between Pb and Ni contents. Additionally, the translocation factors (TF_{soil}, TF_{root}, and TF_{shoot}) and bioaccumulation factor (BAF) above 1 of the rice plant were observed in order of Zn>Fe>Mn>Ni or Pb, Zn or Ni> Pb or Mn >Fe, Pb>Zn or Mn or Fe>Ni, and Zn>Pb>Mn>Ni>Fe, respectively. These data support developing a useful biomarker to monitor organophosphorus and carbamate insecticides and heavy metals, and management programs in health promotion and toxic surveillance for insecticides and heavy metals in food and environments.

Keywords: Bioaccumulation, heavy metals, organophosphorus and carbamate insecticides, rice

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INTRODUCTION

Nakhon Nayok is one of central provinces of Thailand where there are areas for rice growing of approximately 935.3 square km which provide 311,566 tons of rice products (National Statistical Office [NSO], 2014). Ongkharak is one of the most important districts of Nakhon Nayok province in rice cultivation. Nowadays, demand of agricultural products has continued to increase overtime because of Thailand's dramatically growing population. The use of pesticides is therefore essential and rapidly increasing. Previously, it has been reported that pesticides were imported to Thailand in 2016, which was higher than in the previous year by approximately 7.54% (Office of Agricultural Economics [OAE], 2017).

Generally, several chemicals used in paddy fields (i.e. inorganic fertilizers and pesticides) consist of heavy metals (Gimeno-García et al., 1996; Wuana & Okieimen, 2011). Therefore, the application of pesticides can lead to extensive distribution and long-term accumulation of heavy metals in soils (Jablonowski et al., 2012). Heavy metals from soils can enter water reservoirs, then accumulate in plants and animals, and transfer to humans *via* food webs (Gall et al., 2015). Heavy metals are toxic to human health as they directly effect biochemical and physiological functions in the human body (Jaishankar et al., 2014). Moreover, prevalence rates of toxic effect of pesticides have been reported at 14.47 cases per 100,000 people or 8,689 cases per year in 2016 (Bureau of Occupational and Environmental Diseases [BOED], 2016). Recently, insecticides of the organophosphorus and carbamate group are increasingly used to control pests, such as insects in agriculture regions. However, these compounds can impact the environment and human health.

The organophosphorus and carbamate insecticides (e.g., dichlorvos, parathion, fenamiphos, and N-methyl carbamates) are known as irreversible inhibitors of acetylcholinesterase activity and commonly used as biomarkers for environmental and human monitoring (Lionetto et al., 2013; Vale & Lotti, 2015). Corresponding with previous reports, the estimation of acetylcholinesterase inhibitory activities has been used to monitor or indicate the presence of organophosphorus and carbamate insecticides in vegetable and fruit juice (Korpraditskul et al., 2004). Similarly, the relationship between the use of the insecticides and the level of acetylcholinesterase in the blood has been significantly found in 236 farmers from Sam Chuk district, Suphan Buri province (Duangchinda et al., 2014).

Therefore, our major goals focused on monitoring organophosphorus and carbamate insecticides by anti-acetylcholinesterase activity, and measuring heavy metal content in soil, water, and rice from paddy fields in Ongkharak district, Nakhon Nayok province by an atomic absorption spectrometry. Bioaccumulation factor (BAF) and translocation factors (TFs) were also calculated for each rice sample and heavy metal. Our hypothesis was that

percentages of anti-acetylcholinesterase activities correlated with each heavy metal content in rice plants. These basic data are useful for promoting awareness and surveillance of human health and environmental impacts. These benefits help to reduce the insecticide use of farmers in paddy fields, and to manage programs in sustainable prevention and treatment of human diseases from insecticides and heavy metals in food.

MATERIALS AND METHOD

Chemicals

Acetylthiocholine iodide was purchased from Sigma. Acetylcholinesterase enzyme, DTNB (5,5′-dithiobis, 2-nitrobenzoic acid) and nitric acid were obtained from Sigma-Aldrich. Absolute ethanol was obtained from Merk. Heavy metal standards (copper, lead, ferrous, nickel, zinc, manganese, and chromium) for an atomic absorption spectrometry were obtained from Sigma-Aldrich.

Sampling

Samples from paddy soils (*N*= 30), rice (*N*=30) and water (*N*=10) were collected from 10 paddy fields located in Tambon Ongkharak (OK), Khlong Yai (KY), Sisa Krabue (SK), Bang Luk Suea (BLS), Sai Mun (SM), Bang Plakot (BP), Chumphon (CP), Bueng San (BS), Phra Achan (PA), and Bang Sombun (BSB) of Ongkharak district, Nakhon Nayok province between August 2017 and February 2018. Sampling locations were demonstrated with GPS (Global Positioning System) using Google Earth (version 7.1.2.2041) (Figure 1).

Water samples were collected by the grab sampling method at approximately 15 cm depth from the water surface, then kept in

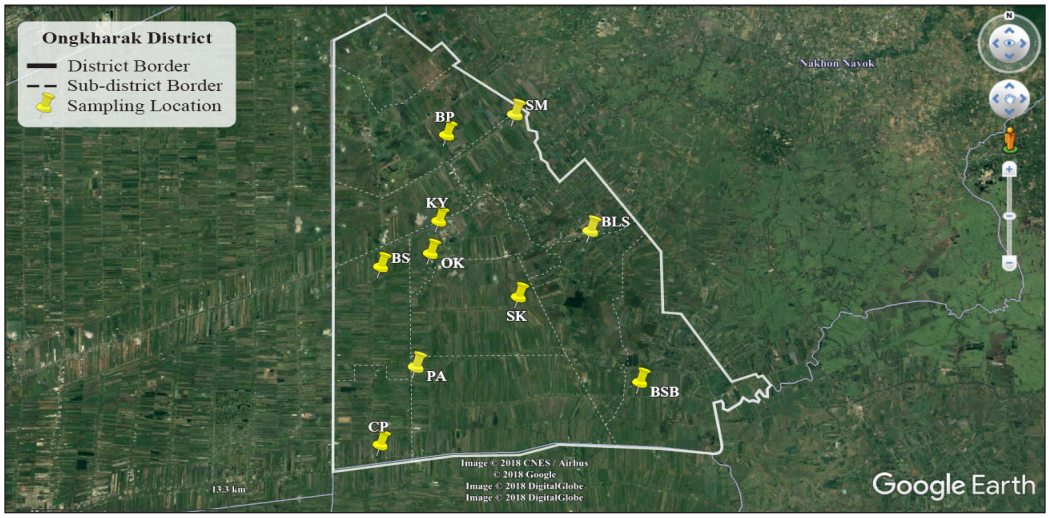


Figure 1. Sampling locations were revealed with GPS (Global Positioning System) using Google Earth (version 7.1.2.2041). Tambon Ongkharak = OK, Khlong Yai = KY, Sisa Krabue = SK, Bang Luk Suea = BLS, Sai Mun =SM, Bang Plakot = BP, Chumphon =CP, Bueng San =BS, Phra Achan =PA, and Bang Sombun =BSB.

50 ml polyethylene tubes at 4°C (Chowdhur et al., 2012; Thummajitsakul et al., 2015). Soil subsamples from 10 sites were collected at 15 depth from the surface by sampling tools, then combined into one sample, and each sample (approximately 500 grams) was used for drying at 50°C before preserving in plastic bags at a temperature of 4°C (Fery & Murphy, 2013; Thummajitsakul et al., 2015). For rice sampling, each subsample was collected from 10 sites per a paddy field, then combined into one sample, and divided into three parts (root, shoot and grain) according to the report of Kingsawat and Roachanakanan (2011). The different parts of the rice plant were cleaned with deionized water, then sectioned into small pieces, and dried at 50°C, followed by grinding with a homogenizer and preserving the powdered samples in plastic bag at 4° C.

Anti-acetylcholinesterase Activity

The anti-acetylcholinesterase activity was performed using Ellman colorimetric assay (Ellman et al., 1961) with some modifications. Each powdered sample (0.5 grams for rice samples and 2.5 grams for soil samples) was mixed with 5% ethanol solvent for 5 min, and left at room temperature for 15 min. Each soil solution was diluted 10 fold. Then, the extract or water sample (200 µl) was mixed with 15 mM acetylthiocholine iodide (200 µl), 3 mM DTNB (1 ml), and 0.3 U/ml of AChE enzyme (200 µl), and then incubated at 37 °C for 15 min. Sample extraction and reaction were performed in duplicate using the same method. An absorbance was determined at 410 nm by spectrophotometer (Model T60UV). Negative controls for quality control procedures were carried out using distilled water, and each sample extract without acetylcholinesterase enzymes. Methylcarbamate was used as positive control at concentrations 0, 0.2, 2, 20 and 200 µg/ml. Percentages of acetylcholinesterase inhibition were calculated following the below formula.

$$\textbf{\%}\ \text{Acetylcholinesterase inhibition} = \frac{[(A_{water} - A_{blank1}) - (A_{sample} - A_{blank2})]*100}{(A_{water} - A_{blank1})}$$

Note:

(a) A_{water} and A_{sample} were the absorbance of distilled water and sample extract with acetylcholinesterase enzyme, respectively.

(b) A_{blank1} and A_{blank2} were the absorbance of distilled water and sample extract without acetylcholinesterase enzyme, respectively.

Digestion and Sample Analysis

Sample digestion was performed according to Kingsawat and Roachanakanan (2011) and Thummajitsakul et al. (2018). The powdered samples of root, shoot, grain and soil (0.5 g), and water sample (50 ml) were digested with 10 ml of 70% HNO_3 (V/V) at 100°C on a hotplate until dried. After that, each digested sample was added to 1% HNO_3, then filtered through Whatman No. 1 paper and the volume adjusted to 50 ml with 1% HNO_3. The digestion process for each sample was performed in triplicate.

Heavy metals Cu, Pb, Fe, Cr, Ni, Mn and Zn were then detected by an atomic absorption spectrometry (Model 200 Series AA, Agilent Technologies). The 1% HNO_3 was used as a negative control. External standard solution at 1000 µg/ml concentration was diluted with 1% HNO_3, and used to generate linear standard calibration curves.

Statistical Analysis

Descriptive statistics (i.e. percentages, mean and SD, etc.) were used for anti-acetylcholinesterase activities and heavy metal contents. Principle component analysis (PCA) and Pearson' correlation were performed to analyze the relationships between % acetylcholinesterase inhibition and heavy metal concentrations of paddy soils, water, and different parts of the rice plant. All statistical analysis was done using the PSPP program version 0.10.5 (Pfaff et al., 2013), and Paleontological statistic program version 3.16 (Hammer et al., 2001). Moreover, bioaccumulation factor (BAF) and translocation factors (TFs) were calculated for each rice sample and heavy metal by formulas below:

$$BAF = C_{grain}/C_{soil}$$
$$TF_{soil} = C_{root}/C_{soil}$$
$$TF_{root} = C_{shoot}/C_{root}$$
$$TF_{shoot} = C_{grain}/C_{shoot}$$

where C_{soil}, C_{grain}, C_{shoot} and C_{root} were concentrations of heavy metals in soil, rice grain, rice shoot and rice root (mg/kg), respectively. The TF_{soil}, TF_{root}, and TF_{shoot} above 1 indicate effectively metal translocation from soil to root, root to shoot, or shoot to grain, respectively. Similarly, if BAF exceeds 1, rice is recognized as hyperaccumulator which absorbed and accumulated heavy metals from the soils (Satpathy et al., 2014; Neeratanaphan et al., 2017).

RESULTS AND DISCUSSION

This work focused on monitoring organophosphorus and carbamate insecticides by determining anti-acetylcholinesterase activities, and heavy metals in paddy field soils, water and rice samples by an atomic absorption spectrometry. The results showed that the percentages of anti-acetylcholinesterase activities were observed in the following order: shoot (72.74 -93.25%) > soil (66.88 -93.10%) > root (61.42 - 93.82%)> grain (26.51-89.66%)> water (5.39 - 76.01%) (Table 1).

Previously, it has been reported that pesticides are not found only in the plant outside, but they can be absorbed, redistributed and accumulated into the tissue of rice plants (Ge et al., 2017). For example, insecticides in organochlorine, organophosphate and synthetic pyrethroid groups can be transferred and accumulated into tomato, pineapple and mango fruits (Agyekum et al., 2015). However, the level of acetylcholinesterase inhibition of samples may be affected by other factors, such as natural acetylcholinesterase inhibitors from plants (i.e. alkaloid, terpenoids, flavonoids and phenolic agents) (Murray et al., 2013), and certain heavy metals Cd, Cu, Zn and Hg *in vitro* (Frasco et al., 2005).

Table 1
Percentages of acetylcholinesterase inhibition of water, soil, rice shoot, rice grain, and rice root samples in agricultural regions of Ongkharak district, Nakhon Nayok Province

Sampling location	% Acetylcholinesterase inhibition (mean±SD)				
	Water	Soil	Shoot	Grain	Root
KY	31.61±7.90	74.93±10.88	72.74±16.39	70.55±21.48	61.42±32.91
SK	56.03±12.35	82.40±7.94	76.01±15.49	80.60±17.63	66.52±14.90
BLS	42.67±3.72	66.88±21.29	76.58±15.59	87.28±9.76	85.06±11.39
SM	5.39±1.74	73.49±19.61	89.94±5.67	84.55±9.48	71.21±15.90
BP	17.39±5.23	75.14±18.67	73.36±25.48	74.50±8.66	75.29±12.39
CP	32.54±3.73	89.44±5.02	80.60±16.41	26.51±4.60	87.36±1.18
BS	76.01±5.51	78.30±13.85	75.65±11.16	64.08±21.22	84.20±8.71
OK	38.36±6.17	89.66±6.31	89.73±9.98	75.14±12.39	93.82±1.61
PA	27.01±11.80	77.30±19.59	93.25±1.00	89.66±5.81	85.78±7.35
BSB	25.29±6.00	93.10±3.27	89.37±12.09	39.80±12.86	82.04±18.77
Total	**36.39±20.00**	80.06±15.35	81.11±15.06**	69.09±25.26**	77.60±20.37

In this study, all heavy metals were found in all samples, except for Cr that was not detected in any samples and Cu that was detected in only one rice sample. The heavy metals were found in the paddy field soils, water, shoot, grain and root of rice plants in order of Fe>Mn>Ni>Pb>Zn. The amount of each heavy metal was mainly higher in root, shoot and grain of rice plants than that of soil and water samples (Table 2). Although, concentrations of heavy metals in soil samples did not exceed the maximum permissible level of each heavy metal, concentrations of Fe, Ni and Pb in all water and rice samples did exceed maximum permissible levels (Table 3).

Table 2
Concentration of each heavy metal found in water, soil, shoot, grain and root from 10 sampling locations of paddy fields

Heavy metals	Concentration of each heavy metal (mean±SD) (mg/kg for rice and soil, mg/l for water)				
	Water	Soil	Shoot	Grain	Root
Mn					
Min	0.32±0.22	70.00±7.78	67.78±3.14	24.44±12.02	40.28±33.78
Max	1.40±0.15	127.41±37.39	1,109.00±279.76	349.26±59.23	154.44±40.18
Mean	1.03±0.26	100.19±18.37	219.19±50.47	102.80±25.60	105.18±22.28
Zn					
Min	0.04±0.02	14.65 ± 7.72	17.00±11.75	12.53±6.09	18.08±8.47
Max	0.16±0.11	29.38±5.43	175.74±151.23	116.91±5.72	49.16±5.96
Mean	0.10±0.06	20.24±8.27	42.59±24.78	39.86±8.36	26.50±7.17

Table 2 *(continue)*

Heavy metals	Concentration of each heavy metal (mean±SD) (mg/kg for rice and soil, mg/l for water)				
	Water	Soil	Shoot	Grain	Root
Ni					
Min	0.34±0.25	66.58±41.67	30.76±25.27	49.61±31.97	28.53±27.35
Max	1.08±0.70	117.48±73.09	578.15±54.19	106.17±45.96	113.37±17.28
Mean	0.77±0.51	89.15±53.22	131.31±44.37	75.40±54.34	77.70±36.75
Fe					
Min	4.67±3.25	176.19±33.67	673.33±321.46	534.29±132.92	10,952.38±5,569.60
Max	111.35±59.98	40,476.19±30,799.38	5,904.76±2147.26	1,562.86±852.25	29,309.52±4,592.79
Mean	19.82±9.95	14,968.87±7549.43	1,515.59±592.75	876.15±477.94	17,216.55± 6951.86
Pb					
Min	0.31±0.14	26.80±21.17	22.8±11.31	12.80±8.48	19.80±13.61
Max	0.74 ±0. 6 3	77.20±38.74	78.00±17.98	94.80±39.60	82.80±50.79
Mean	0.46±0.29	51.85±35.62	54.97±25.52	65.24±39.32	51.29±34.00
Cu					
Min	ND*	ND*	ND*	ND*	ND*
Max	ND*	ND*	ND*	ND*	ND*
Mean	ND*	ND*	ND*	ND*	ND*
Cr					
Min	ND*	ND*	ND*	ND*	ND*
Max	ND*	ND*	ND*	ND*	ND*
Mean	ND*	ND*	ND*	ND*	ND*

*ND is not detected.

Table 3
Maximum permissible levels for each heavy metal and % samples from 10 sampling locations that showed concentrations above the maximum permissible levels

	Mn	Zn	Ni	Fe	Pb
*MPL_{soil}	1,800[a]	7,500[b]	1,600 [a]	20,000-550,000[b]	400[a]
*MPL_{water}	1.0[c]	1.0[c]	0.1[c]	0.5[d]	0.05[c]
*MPL_{rice}	NV[g]	100[e]	1[f]	20[e]	0.5[e]
% samples > *MPL_{soil}	0	0	0	0	0
% samples > *MPL_{water}	60	0	100	100	100
% samples > *MPL_{rice}	-	10	100	100	100

* MPL = Maximum permissible levels for heavy metal (mg/kg for soil and rice samples, mg/l for water samples)
[a] Ministry of Natural Resources and Environment [MNRE], Thailand (2004)
[b] Data from Chawpaknum et al. (2012)
[c] Ministry of Natural Resources and Environment [MNRE], Thailand (1994)
[d] Department of Groundwater Resources [DGR], Thailand (2008)
[e] Ministry of Public Health [MOPH], Thailand (1986)
[f] United States Department of Agriculture - Foreign Agricultural Service [USDA – FAS] (2014)
[g] NV is the no criterion value

Moreover, correlations between heavy metal contents and % acetylcholinesterase inhibition of all investigated samples were analyzed. Interestingly, the strongly and moderately positive correlations between % anti-acetylcholinesterase activities and concentrations of Pb and Zn were respectively found, significantly. Additionally, the % anti-acetylcholinesterase activities showed weakly positive correlation with Mn, Ni, and Fe contents, significantly. Therefore, this study implied that an increase of insecticide levels in water, soil and rice samples was associated with higher concentration of heavy metals. Moreover, very strongly positive correlation was observed between Zn and Mn, while Zn showed moderately positive correlation with Pb, significantly. The Pb content also showed weakly positive correlation with concentrations of Mn and Ni contents, significantly (Table 4). It implied that an increase of Zn concentration was associated with higher concentrations of Mn and Pb in the samples. Similarly, an increase of Pb concentration correlated with the presence of higher concentrations of Mn and Ni in the samples.

The organophosphorus and carbamate chemicals are insecticides that inhibit acetycholinesterase enzymes (Vale & Lotti, 2015). Therefore, determination of anti-acetylcholinesterase activities can be used to link the level of organophosphorus and carbamate insecticides in targeted samples (Korpraditskul et al., 2004). In this study, it showed that the presence of the Pb, Zn, Mn, Ni, and Fe heavy metals might help either to stabilize the insecticides used in paddy fields, or the insecticides composed of the heavy metals. Similarly, Zn contributed to the Mn and Pb stabilization, and Pb facilitated the Mn and Ni stabilization. Similarly, previous report revealed a positive correlation between acetamiprid insecticides and Cu, and indicated that Cu facilitated the stabilization of acetamiprid insecticides (Tariq et al., 2016).

Table 4
Pearson's correlation matrix between the percentages of acetylcholinesterase inhibition and each heavy metal, and between heavy metal pair

	% AChE inhibition**	Mn	Ni	Pb	Zn	Fe
% AChE inhibition	1.00					
Mn	0.361*	1.00				
Ni	0.330*	0.043	1.00			
Pb	0.680*	0.370*	0.372*	1.00		
Zn	0.423*	0.870*	0.116	0.527*	1.00	
Fe	0.337*	0.067	0.120	0.171	0.018	1.00

*Correlation was significantly found at $P < 0.05$
**% AChE inhibition is the percentages of acetylcholinesterase inhibition

Furthermore, bioaccumulation factor (BAF) and translocation factors (TFs) were analyzed. The results showed that the order of BAF values of heavy metals was Zn>Pb>Mn>Ni>Fe. The BAF > 1 of grain samples indicated that the rice plant was able to accumulate the heavy metals, especially Pb and Zn (Table 5). The transfer factors (TFs) of heavy metals from soil to root (TF_{Soil}), root to shoot (TF_{Root}), and shoot to grain (TF_{Shoot}) were also analyzed. The averages of TF_{soil}, TF_{root}, and TF_{shoot} were demonstrated in the following order: Fe>Zn>Mn>Pb>Ni, Mn>Ni>Zn>Pb>Fe, and Pb>Zn>Ni>Fe>Mn, respectively (Table 5). It indicated that translocation of Fe, Mn and Pb from soil to root, root to shoot, shoot to grain was greater than other heavy metals, respectively. The concentrations of Fe, Mn and Pb revealed the highest values in root, shoot and grain, respectively. Pb was highly accumulated in grain corresponding to the BAF above 1. The sample percentages, which there were TF_{soil}, TF_{root}, TF_{shoot} and BAF values above 1, were shown in the order of Zn>Fe>Mn>Ni or Pb, Zn or Ni> Pb or Mn >Fe, Pb>Zn or Mn or Fe>Ni, and Zn>Pb>Mn>Ni>Fe, respectively (Table 6). However, all heavy metals could be transferred from soil and accumulated in the grain of rice plants. The pathway of heavy metal translocation in plants is a major component in heavy metal redistribution through different parts of the plants (Page & Feller, 2015).

Similarly, it has been reported that Zn, Cd and Cu are mostly found in paddy field soils, water, and different parts of rice plant, namely root, shoot, grain and husk (Kingsawat & Roachanakanan, 2011). Corresponding to the previous report, heavy metals (Cd, Cr, Pb, Zn, As, Mn, and Hg) were higher in root than straw, and grain of rice plants (Singh et al., 2011). Additionally, it has been reported that Cd, Pb, Ni, and Zn in rice crop (*Oryza sativa* L.) were highly accumulated in rice stem and grain (Rahimi et al., 2017).

In Thailand, it has been reported that concentrations of heavy metals (Cd, Cr, Pb, Cu, Ni and Zn) in paddy field soils for organic rice cultivation are much below maximum permissible levels of the heavy metals (Chinoim & Sinbuathong, 2010). The uptake and accumulation of certain heavy metals (Pb, Cd, Fe, Cr, Ni, and Zn) into Thai jasmine rice in paddy field soil from Mea Sod in Thailand have been reported that Pb, Cd and Fe are mostly accumulated in roots, and Cr, Ni and Zn are mostly accumulated in stems and grains (Thongsri et al., 2010). Concentrations of soil heavy metals (Zn, Cd and Pb) influence contents in different parts of rice according heavy metal concentrations in soil, and accumulation of heavy metals is found higher in root than grain and straw (Roongtanakiat & Sanoh, 2015).

In this study, the principle component analysis was used to demonstrate correlations among % acetylcholinesterase inhibition and heavy metal contents by principle components 1 and 2 that showed 46.4% and 21.8% of total variance, respectively. The Mn, Pb and Zn contents and % anti-acetylcholinesterase activities

Table 5
*Translocation factors (TF_{Soil}, TF_{Root}, and TF_{Shoot}) and bioaccumulation factor (BAF) of the rice plant (*Oryza sativa *L.)*

Heavy metals \ Sampling locations	KY	SK	BLS	SM	BP	CP	BS	OK	PA	BSB	Mean±SD
Mn											
TF_{soil}	1.29	0.93	1.01	1.02	0.33	2.00	0.82	1.45	1.13	0.82	1.08±0.44
TF_{Root}	2.22	1.57	1.04	9.33	3.45	0.65	0.87	0.59	0.85	1.17	2.17±2.66
TF_{Shoot}	0.24	1.16	0.21	0.31	0.28	0.60	0.34	1.26	2.02	0.46	0.69±0.60
BAF	0.68	1.69	0.22	3.01	0.33	0.78	0.24	1.09	1.96	0.45	1.04±0.91
Zn											
TF_{soil}	1.32	1.11	0.62	1.73	3.36	1.57	0.84	1.00	1.47	1.14	1.42±0.76
TF_{Root}	1.21	2.01	0.94	5.19	0.40	1.12	1.27	1.54	0.73	1.44	1.58±1.34
TF_{Shoot}	0.71	1.54	3.39	0.67	1.11	0.85	0.47	0.99	0.97	0.75	1.14±0.84
BAF	1.14	3.44	1.96	5.97	1.48	1.49	0.50	1.52	1.04	1.23	1.98±1.60
Ni											
TF_{soil}	0.84	0.43	0.34	0.58	1.11	1.27	1.14	1.09	1.32	0.79	0.89±0.35
TF_{Root}	0.35	2.73	3.49	1.02	0.61	0.79	5.84	1.04	1.07	1.40	1.83±1.40
TF_{Shoot}	2.22	0.64	0.59	0.79	0.89	1.24	0.18	0.87	0.92	0.82	0.91±0.82
BAF	0.65	0.75	0.71	0.47	0.60	1.25	1.18	0.98	1.30	0.90	0.88±0.90
Fe											
TF_{soil}	0.46	1.41	0.93	1.64	1.35	64.68	1.68	1.74	1.15	0.65	7.57±20.07
TF_{Root}	0.05	0.04	0.09	0.29	0.05	0.09	0.04	0.05	0.09	0.11	0.09±0.08
TF_{Shoot}	0.75	1.20	1.04	0.14	0.69	0.98	0.78	0.55	1.24	0.60	0.80±0.33
BAF	0.02	0.07	0.08	0.07	0.05	5.50	0.05	0.04	0.12	0.04	0.60±1.72
Pb											
TF_{soil}	1.49	1.27	0.79	2.33	0.34	0.64	1.13	0.71	0.82	1.37	1.09±0.57
TF_{Root}	0.57	1.40	1.05	0.94	3.85	1.26	1.19	1.12	0.66	0.73	1.28±0.94
TF_{Shoot}	2.27	1.30	1.78	1.03	0.98	0.38	0.80	0.76	2.02	1.33	1.27±0.60
BAF	1.93	2.32	1.48	2.27	1.29	0.30	1.08	0.61	1.10	1.33	1.37±0.66

Table 6
Percentages of rice samples that there were TF and BAF values above 1 for each heavy metal

	% of rice samples				
	Mn	Zn	Ni	Fe	Pb
$TF_{soil} > 1$	60	80	50	70	50
$TF_{Root} > 1$	60	70	70	0	60
$TF_{Shoot} > 1$	30	30	20	30	60
BAF > 1	40	90	30	10	80

were clustered in PC1, and Ni and Fe in PC2 on positive view of the PCA biplot. The result showed that the PCA biplot confirmed positive correlation of Fe, Ni, Pb, Zn, Mn accumulation and % anti-acetylcholinesterase activities in shoot, grain and root of the rice plant (Figure 2A and 2B).

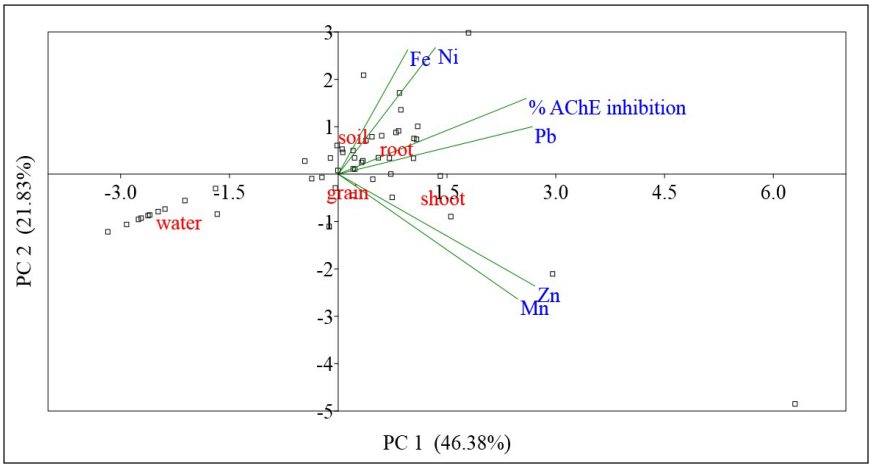
There are, however, still other factors (i.e. plant species, bioactive agents and metal category) that can effect on the BAF and TF values of plants (Balabanova et al., 2015; Page & Feller, 2015). Moreover, all samples in this study were collected from 10 sampling locations in Ongkharak district of Nakhon Nayok province. Among these sampling locations, the public landfill is located in Tambon Sai Mun, which accepts enormous quantities of waste from other areas in Nakhon Nayok province, such as Srinakharinwirot University, Ongkharak (Yunak et al., 2016). A landfill area is an important factor of heavy metal contamination in soils and plants (Chuangcham et al., 2008; Kasam et al., 2018), especially if disposing of hazardous materials. The large amount of spent household batteries discarded in landfills cause infiltration of higher heavy metals, such as Mn and Zn, into surroundings (Karnchanawong & Limpiteeprakan, 2009).

Heavy metals can, therefore, accumulate from natural sources or biogeochemical cycles in geological structures of the earth, or from anthropogenic sources (Garrett, 2000; Satpathy et al., 2014). They can accumulate in water resources *via* natural methods, such as rainfall, flowing and infiltrating of water into canals and soils. Generally, heavy metals are deposited on the upper part of soils, and accordingly the top soils are frequently used to monitor heavy metals occurring from human activities. Additionally, heavy metals from soils can affect the ecosystem and human health by entering into groundwater, or transferring to plants (Bhagure & Mirgane, 2011).

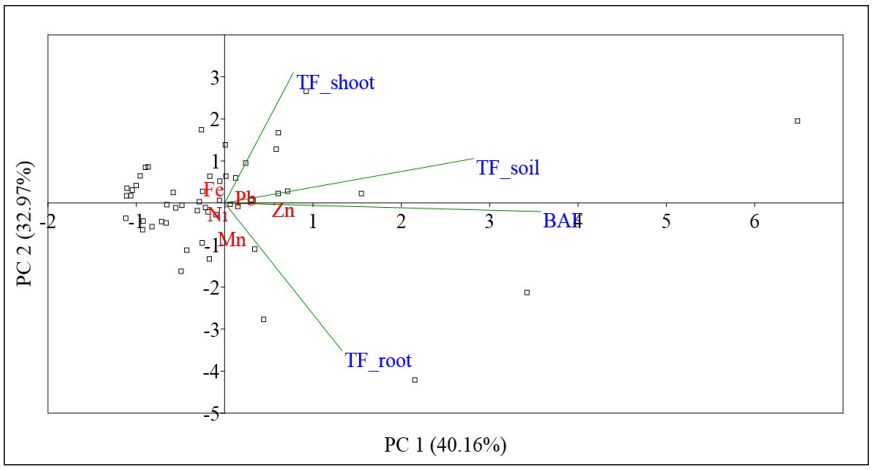
Several previous studies have reported that the application of fertilizers and pesticides in paddy fields can release certain toxic heavy metals (Zn, Mn, Cd, Cu, Cr and Pb) into soil, which can be transferred into different parts of rice plant (*Oryza sativa* L.), especially grains (Satpathy et al., 2014). Moreover, insecticides, fertilizers, herbicides and fungicides are major sources of heavy metals, such as Cu, Cd, Pb, Co, Ni, Zn, Fe and Mn (Chiroma et al., 2007; Gimeno-García et al., 1996; Tariq et al., 2016).

Thus, the uncontrolled application of pesticides in paddy fields can cause several environmental problems, such as heavy metal contamination in plants, soil and water (Kingsawat & Roachanakanan, 2011). Moreover, the long-term and extensive use

of pesticides can also effect human health *via* the food web, and working or living near farms and industries (Al-Saleh & Abduljabbar, 2017; Jaga & Dharmani, 2003; Mahmood & Malik, 2014). Heavy metals cause poisoning to kidney, lung, liver and brain of humans by activating oxidative stress and inhibiting enzymes in metabolic processes which can cause mutations and cancers (Jadoon & Malik, 2017; Morales et al., 2016; Sharma et al., 2014).



(A)



(B)

Figure 2. Principle component analysis for heavy metal concentrations and the percentages of acetylcholinesterase inhibition of water, soil, rice shoot, rice grain, and rice samples from different sampling sites (A), and for bioaccumulation factor (BAF), translocation factors (TFs), and heavy metals of the samples (B)

CONCLUSION

In the current studies, organophosphorus and carbamate insecticides in water, soils and different parts of the rice plant (shoot, grain and root) were monitored by determining acetylcholinesterase inhibitory activities. The order of % anti-acetylcholinesterase activities was shoot > soil > root > grain > water, and the order of heavy metal contents was Fe>Mn>Ni>Pb>Zn. The Fe, Mn, Ni, Pb and Zn contents of soil samples were below maximum permissible levels, but the Fe, Ni and Pb contents of all water and rice samples were above maximum permissible levels. Moreover, the percentages of anti-acetylcholinesterase activities correlated positively with concentrations of Pb, Zn, Mn, Ni, and Fe, significantly. Positive correlations were also significantly found between Zn and Mn, between Zn and Pb, between Pb and Mn, and between Pb and Ni. The order of TF_{soil}, TF_{root}, TF_{shoot} and BAF values which exceeded 1 was Zn>Fe>Mn>Ni or Pb, Zn or Ni> Pb or Mn >Fe, Pb>Zn or Mn or Fe>Ni, and Zn>Pb>Mn>Ni>Fe, respectively. These useful data may be applied to develop a potential biomarker for monitoring organophosphorus and carbamate insecticides and heavy metals in food and environments, and to manage programs for human health promotion to avoid toxicity from the insecticides and heavy metals.

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Effect of *Lantana camara* L. and *Parthenium hysterophorus* L. to Control Pathogenic Nematode, *Meloidogyne incognita* (Kofoid and White) Chitwood

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ABSTRACT

Lantana camara L. and *Parthenium hysterophorus* L. are two invasive weed species in Malaysia, but sufficient information is not available on the uses of these invasive species for producing value-added products. Therefore, the plant extracts of these species were tested against the pathogenic nematode, *Meloidogyne incognita* to explore the possibility of using these species in the industry for commercial production of bionematicide. Aqueous extracts of the weed species were made at the Universiti Malaysia Kelantan (UMK) laboratory by mixing 0 g, 10 g, 20 g, and 40 g dried plant powder with 100 ml distilled water. The plant extracts (6 ml) at four different concentrations, e. g. 0%, 10%, 20% and 40% were added to the nematode suspension containing 50.33±2.52 Juveniles in urine jars, and the contents were kept undisturbed for 24 hours. The number of dead nematodes was counted by placing the treated extracts on the microscopic slide and was observed under a compound microscope. The data revealed that both the species had the killing effect on the nematode. Between two plant species, *L. camara* was more effective causing 83% mortality at 40% concentration of extracts. *P. hysterophorus* caused 81.5% mortality of the nematode at the same concentration (40%). The plant species might be the raw materials for producing bionematicide in the industry, and the effective concentration might be reduced by purifying and partitioning the crude extracts with appropriate solvents and techniques.

Keywords: Bionematicide, *Lantana camara*, *Meloidogyne* incognita, *Parthenium hysterophorus*, root-knot nematode

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INTRODUCTION

Lantana camara is an invasive weed and a shrub usually found on the roadside and fallow land, but sometimes it is used as a flowering ornamental plant. The plant is reported to have medicinal properties such as antibacterial activity, cytotoxic activity, antifertility, antifungal activity, anti-inflammatory activity, antimotility activity, antidiabetic activity, larvicidal activity, antioxidant activity, wound healing activity and hepatoprotective effects (Ghisalberti, 2000; Kalita et al., 2012).

Parthenium hysterophorus is another invasive alien species in many countries of Asia, Africa and America (Seema, 2011). It has many negative impacts on human and animal health, crop production, and biodiversity. In Malaysia, the weed has been identified as an invasive, allergenic and environmental pollutant (Karim, 2014; Karim et al., 2017). However, the weed is reported to possess some medicinal and pesticidal properties as well (Kumar et al., 2013).

Most of the nematodes are well known as a plant-parasitic organism and the species, *Meloidogyne incognita* is an important plant-parasitic nematode which affects the quality and quantity of the crop production including banana, tomato, pulse and many vegetables. This nematode multiplies mainly due to the completion of several generations within a single growing season. According to Al-Hazmi et al. (2017), the species, Meloidogyne are responsible for the reduction of crop yield by 10% in vegetable crops. In some places the crop losses extend to 30%, depending on the host cultivar, population density and the species of nematode that involves. Therefore, bio-control of this parasite is explicitly essential for sustainable crop production (Stoffelen et al., 1999). The usual practice of control is the use of artificial chemical, e.g., Methyl bromide, which leads to environmental pollution. The use of botanical to control this pest is more eco-friendly and sustainable. Udo et al. (2014) studied the effect of *Paecilomyces lilacinus* in combination with *Lantana camara* leaf extract in controlling root galling of tomato where they noticed that *L. camara* with *P. lilacinus* were effective in reducing egg production of *M. incognita*. However, the killing effects of the extract of *L. camara* on the *M. incognita* extracted from banana were not targeted in their study. The plant extract of *L. camara* and *P. hysterophorus* are allelopathic and might be effective botanicals against the root-knot nematode. Unfortunately, sufficient information on this aspect is not available in Malaysia.

The objective of the study was to assess the nematicidal effect of plant extracts of *L. camara* and *P. hysterophorus* on *M. incognita.*

MATERIALS AND METHODS

The leaves of *Lantana camara* were collected from the roadside of Bera, Pahang while that of *Parthenium hysterophorus* were collected from Baling, Kedah. The nematode, *M. incognita* was collected from University of Malaya (UM) where these were reared in the experimental soil

of banana plantation. The infected banana plants along with contaminated soil were carried to Universiti Malaysia Kelantan (UMK) AgroTeck Park, Jeli Campus. The collected banana plants were grown at AgroTeck Park until the Juveniles were used in the study.

The nematodes along with root soil of infected banana plants were collected from AeroTech Park. The soil around the banana suckers contained the test nematodes, *M. incognita.* The 'Baermann techniques' was used for extraction of active nematodes from the soil. The soil was removed from the roots of the banana plants, and a container was used to extract the nematode. A small handful of infected soil was overlapped on two layers of wet facial tissues. The wrapped soils were placed in a small porous dish on the top of a mesh. Water was added gradually so that the mesh is slightly covered with water and the soil become wet. After 24 hours rest, the nematodes were crawled out of the soil. The container was covered with plastic wrap to prevent from drying. The bundled soil was removed from the container, and the water in the container was examined using a compound microscope. Before using the nematode suspension for testing against the plant extracts, the number of nematode per unit volume of suspension was counted following the standard procedure of Chedekal (2013). Ten ml of suspension was put into a beaker and drop by drop were examined under a microscope until the ten ml of suspension was finished. The presence of nematodes in the suspension drops was confirmed by seeing the thread-like mobile organism in the suspension. This step was replicated thrice. The average number of nematode in 10 ml of suspension was 50.33 ± 2.52.

The early collected fresh leaves of *L. camara* and *P. hysterophorus* were put in paper bags. The collected leaf samples in paper bags were dried in an electric oven at a temperature of 30ºC for three days (Maharjan et al. 2007). The dried leaves were ground into a fine powder using a mechanical grinder. The ground samples were sieved to get a fine powder. The aqueous extract was prepared using distilled water with specified amount of powder, e.g. water (10 g in 100 ml) made 10% solution and so on. After mixing the sample with distilled water, all the solutions were placed into an orbital shaker for 24 hours. Then the extract was first filtered through a clean muslin cloth and then through a filter paper. The filtrates were kept in a refrigerator at 4ºC until it was used for the experiment.

The test was carried out in urine jars. There were 24 urine jars in four replications (2x3x4=24). Ten ml of suspension was taken in a urine jar which contained about 50.33 nematodes. Six ml of early prepared plant extracts of different concentrations of two weed species was added to the suspension in the jars. All the urine jars were kept at ambient temperature. After 24 hours of incubation, all dead and alive 2nd stage juveniles (J2) were counted with the aid of an inverted compound microscope at a magnification of 100x. The dead juveniles attained the shape of a straight line or a bit curved, and the mortality was ensured by

touching the juvenile with a fine needle. The percentage of mortality due to the effect of plant extracts was estimated according to the formula as below:

$$\text{Mortality (\%)} = \frac{\text{(No. of live nematode before treatment)} - \text{(No. of live nematode after treatment)}}{\text{No. of live nematode before treatment}} \times 100$$

The treatments were arranged in a Randomized Block Design, and the data were analyzed using two-way ANOVA by using computerized statistical program SPSS to see significant differences between the plant extracts and their concentrations. The Turkey test was used to compare the means of the treatments at 5% level of significance (Pallant, 2011).

RESULTS AND DISCUSSION

The two-way ANOVA indicates that there was no significant difference between plant species in controlling the nematode, $F\ (1, 20) = 2.840,\ p = 0.107$. Significant difference was noticed among the concentrations, $F\ (3, 20) = 1\ 438.12,\ p = 0.00$. No significant interaction between two factors was also found in the study, $F\ (3, 20) = 0.356,\ p = 0.785$.

It is evident that the application of plant extracts of selected plant species made the significant death of the nematode. All the treatments exhibited nematicidal potential of varying degrees. The mortality of juveniles might be due to nematicidal chemicals present in the leaf extract as *L. camara* contains camaric acid and olenolic acids which may have larvicidal or ovicidal properties (Ghimire et al., 2015). When the dead nematodes were studied under microscopes, it became apparent that they had a variety of shapes. The shape of nematodes before treatment application and after the application was different. Before the application, the shape of nematode was curled (∞-shape) or sigmoid (∑-shape) and bent (banana-shape) while after the treatment application the nematodes were like straight lines (I-shape) and bent banana-shape (Figure 1).

On an average, more than 54% of the nematodes were killed due to treatment with the plant extracts. It means that more than half of the nematodes were died due to the treatments. The number of dead nematodes at 10% concentration of *L. camara* and *P. hysterophorus* were 21.5 and 20.0. The percentage of mortality at 40% concentration for *L. camara* and *P. hysterophorus* are 83.0 and 81.5 (Table 1). The leaf extract of *L. camara* at 100% concentration exposed for 48 hours was found effective in controlling *Meloidogyne* juveniles in Nepal (Ghimire et al., 2015). The nematicidal activity of *L. camara* against juveniles of *Meloidogyne* spp. has also been reported by many authors (Begum et al., 2008; Qamar et al., 2005).

The mortality of the nematode was concentration dependent, and the mortality percentages increased linearly with the concentration. Based on Figure 2,

40% concentration of *L. camara* and *P. hysterophorus* gave the highest mortality compared to other concentrations. The allelopathic plants at higher concentration produced the greater phytotoxic effect on the nematodes.

Figure 1. Characteristics of nematodes before and after extract application

Table 1
Number of live nematodes before and after applying of plant extracts

Concentration of plant extracts	***Lantana camara***			***Parthenium hysterophorus***			**Mean Mortality**
	Before	After	Mortality % ± SD	Before	After	Mortality % ± SD	
0%	50	50	-	50	50	-	-
10%	50	39.25	21.50 ± 1.92	50	40.0	20.00 ± 1.63	20.75
20%	50	18.50	63.00 ± 2.58	50	19.8	61.00 ± 3.46	62.00
40%	50	8.50	83.00 ± 2.58	50	10.0	80.00 ± 2.82	81.50

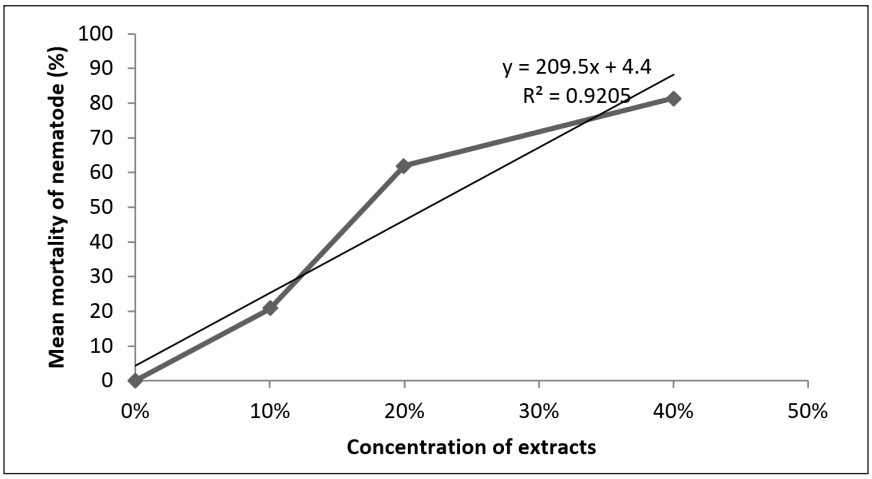

Figure 2. Effect of concentration of extracts on nematode mortality. The values are mean of two weed species (The straight, thin line indicates the linear relationship)

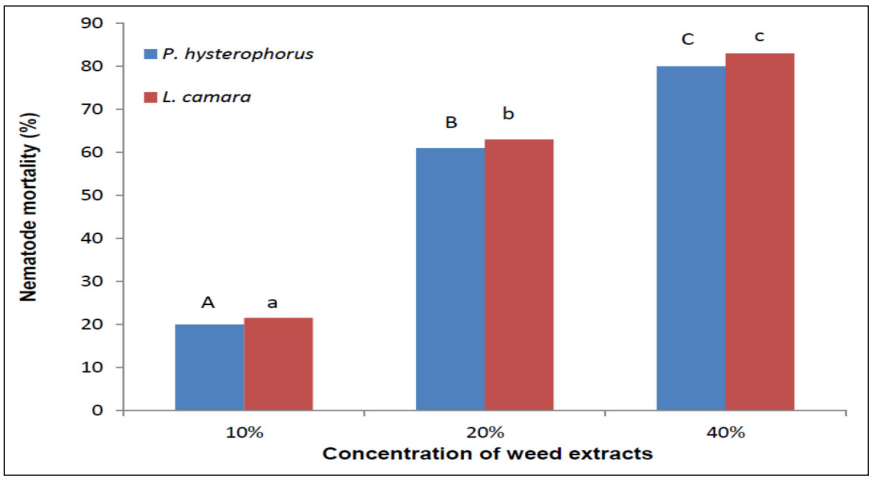

Figure 3. Effects of weed species and different concentrations on the nematode mortality (Dissimilar letters above the red and blue bars indicate significant differences between same colored bars)

The highest nematode mortality (83%) was noticed due to the application of 40% extracts, and there was no significant difference between the plant species at the same concentration (Figure 3). From Figure 3, it is evident that *L. camara* is a bit more effective than *P. hysterophorus* in killing the nematodes. The plant *L. camara* caused 15.9% more mortality than *P. hysterophorus.*

Ghimire et al. (2015) also observed similar kind of killing effect of *L. camara* on *M. incognita*. They stated that the extraction from leaves of *L. camara,* and leaves and root of Mexican marigold could reduce the hatching of *M. incognita* eggs. The inhibitory effect of extracts of the selected botanicals including *L. camara* and *P. hysterophorus* might be due to the

chemicals present in the extracts that possess ovicidal or larvicidal properties. Probably these chemicals affected the embryonic development of the nematode or killed the eggs (Wondimeneh et al., 2013). *L. camara* contains pentacyclic triterpenoids, e.g. coumaric acid, lantanilic acids and olenolic acids which may have larvicidal and ovicidal properties (Ahmad et al., 2010). The properties of *L. camara* and *P. hysterophorus* are also reported to have insecticidal, nematicidal and herbicidal effects in India (Datta & Saxena, 2001; Mishra, 2014).

Wondimeneh et al. (2013) suggested that the nematicidal properties of botanicals were dependent on plant species, plant growth stages, application method and the species of nematode tested. The nematotoxicity of the plant is increased with increase in concentrations and the time of exposure to the nematode. Chaudhary et al. (2013) tested eleven weed species including *L. camara* in Eritrea and observed the least inhibitory effects of *L. camara* on egg hatching of *M. incognita*.

Both the species of plant exhibited toxicity towards the juvenile of the root-knot nematode, *M. incognita*. Usually, when the concentration of plant extract increases the number of nematode mortality also increases. In this study, more than 83% mortality was noted due to 40% concentration of *L. camara*.

However, no concentration greater than 40% was used in this study, and we do not know what could happen if we used more than 40% concentration. But 83% control is an indication of effective control which we could achieve with 40% concentration of the plant extract. More studies should be conducted on the effect of different solvents examples ethanol, methanol, etc., which might give varying degrees of killing effects due to the isolation of different amounts of allelochemicals. The effects of mixing of various plant extracts to control the nematode should also be studied. The blending of plant extracts may provide with a combination of more allelochemicals, which could bring more mortality.

CONCLUSION

In conclusion, the plant extracts of *L. camara* and *P. hysterophorus* can be used to control the plant parasitic nematode *M. incognita*. Using 40% concentration of aqueous extracts of the weeds for more than 80% control of root-knot nematode seems effective botanicals. To produce bionematicide from these weed species the crude extract should be purified and made free from other non-effective compounds, and in that case, a lower concentration of the extracts may be sufficient to control the nematode effectively. The use of these invasive weeds for developing nematicide has explored the possibility of control of the weeds by utilization. The harmful plants could be converted to value-added product. The possibility of establishing SMEs in the country with this technology has also explored a new direction of agro-business.

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Removal of Heavy Metals in Lake Water Using Bioflocculant Produced by *Bacillus subtilis*

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ABSTRACT

Bioflocculant is the extracellular polymeric substances that are produced as by-products of microbial growth with flocculating capabilities. Accordingly, a potential bioflocculant-producing bacterium was isolated from lake water and had been identified as *Bacillus subtilis* (*B. subtilis*). The removal of heavy metals from synthetic wastewater and lake water samples that had been treated with bioflocculant produced by the strain was investigated. Synthetic wastewater samples were prepared by spiking the solution with known concentration of Cd, Cr, Cu whereas the lake water samples had been collected from Cempaka Lake, Bangi, Malaysia. Concentration of the metals in the samples before and after treatment by the bioflocculant was measured using ICP-MS. The removal of heavy metals in synthetic wastewater was found to be ineffective without further pH and dosage manipulations. On the other hand, heavy metals found in the lake water samples (Al, Zn, Fe, Cu) were effectively removed at 92.9%, 94.3%, 86.2% and 68.1% respectively; the treatment was optimised at pH 2 ($p<0.05$), while effects of varying dosages were proven insignificant ($p>0.05$). Bioflocculant produced by *B. subtilis* had been proven to be a good alternative to chemically-based solution in remediating heavy metal polluted waters.

Keywords: B. subtilis, bioremediation, bioflocculant, heavy metals

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INTRODUCTION

Heavy metal pollution is a significant environmental problem. Chemical-intensive industries bearing heavy metals, such as lead (Pb), chromium (Cr), cadmium (Cd), copper (Cu), arsenic (As), zinc (Zn), nickel (Ni) and mercury (Hg) pose threats to human health and the environment through the untreated discharge of their wastewater

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into the aquatic ecosystem (Barakat, 2011). The high solubility characteristics of heavy metals will lead to their absorption by living organisms and bioaccumulate in the food chain, where eventually large concentrations of heavy metals may enter the human body (Low et al., 2015). Ingestion of metals beyond the permitted limit can lead to adverse health side effects (Kooner et al., 2014). In Malaysia, occurrences of heavy metals pollution are being continuously reported all over the country. Sultan et al. (2011) had reported heavy metals pollution had been detected on the surface water of the tropical river watershed in Terengganu. In 2012, Prasanna et al. (2012) and Lim et al. (2012) had reported on the occurrences of heavy metals in the Curtin Lake in Miri and Langat River in Selangor, respectively. Similarly, reports on heavy metals pollutions in Gombak and Penchala River, Selangor had been highlighted by Ismail et al. (2013), while Idriss and Ahmad (2014) had reported on Juru River in Penang, and Al-Badaii and Suhaimi-Othman (2014) report was on Semenyih River, Selangor. Occurrences of heavy metals in the coastal areas and in storm water runoff were also reported by Sany et al. (2013) in Port Klang, Selangor and by Chow et al. (2013), respectively. The most hazardous metals that have been reported includes Pb, Cr, Cd, Cu, As, Cd, Pb, Zn, Ni and Hg (Barakat, 2011). Wastewater from the textile industries (Halimoon & Yin, 2010) and from industrial activities such as burning of fossil fuels, mining, cement manufacturing, paper and glass production and waste recycling (Sany et al., 2013) are among the sources of reported heavy metals pollution. These overbearing occurrences of the metal pollutions in the ecosystem lead to the imminent need for an effective solution (Gaur et al., 2014).

Particulate and soluble form of heavy metal ions can potentially be accumulated by live or dead bacterial cells as well as their by-products (Gupta & Diwan, 2017). Bioflocculants are metabolic by-products of microorganism during growth (Subramanian et al., 2009), and according to Lee and Chang (2018) over the past decades bioflocculant that is produced by many microorganism including bacteria, algae, actinomomyces, fungi and yeast are being used as substitutes for chemical flocculants in water and for wastewater treatment purposes. Bioflocculants have also been described in various studies as potential metal binders (Chen et al., 2016; Gomaa, 2012; Lin & Harichund, 2012; Pathak et al., 2017; Sajayan et al., 2017). In this study, removal of heavy metals by bioflocculants produced by *B. subtilis* that had been isolated from lake water was attempted both on synthetic heavy metal solutions and lake water samples.

MATERIALS AND METHODS

Potential bioflocculant-producing bacteria were isolated from water samples collected from three different lakes in Universiti Putra Malaysia and cultured onto tryptic soy agar (TSA) at 37^{o}C for 24 hrs. Four pure colonies of mucoid and ropy strains were then selected and sub-cultured into tryptic soy broth (TSB), incubated on an orbital shaker

at 150 rpm, 25°C, for 24 h and tested for flocculating activities through kaolin assay. Both the TSA and TSB contain enzymatic digests of casein and soybean as their main ingredients.

The strain with the highest flocculating performance was then chosen and further characterised and identified through 29 biochemical and enzymatic reaction tests (BBL Crystal Gram-Positive ID System). These tests include the hydrolysis of amide and glycosidic bonds with positive utilisation of arginine, positive utilisation of carbohydrates such as glycerol, sucrose and mannitol and positive release of several fluorescent coumarin derivatives.

Flocculation Assay Using Kaolin Clay

Kaolin assay for the determination of flocculating activity was conducted in accordance with the study by Zulkeflee et al. (2012). About 0.5 mL cultured broth and 4.5 mL 0.1% (w/v) $CaCl_2$ were pipetted into 50 mL of 5 g/L kaolin clay suspension. The mixture was then agitated at 200 rpm for 30 seconds and left to stand for 5 mins. The cleared upper phase of the suspension was then collected and measured using a spectrophotometer (Spectronic 20 Genesys, USA) at 550 nm. Flocculating activity is expressed based on the following formula:

$$\text{Flocculating activity (\%)} = [(A-B) / A] \times 100$$

where, A is the optical density of the control and B is the optical density of the sample.

Bioflocculant Source and Timeline of Bioflocculant Production

The selected strain was incubated in tryptic soy broth on an orbital shaker at 150 rpm at 25°C for three days, to determine the timeline of bioflocculant production during growth. The growth of the strain was monitored through optical density measurements of the cultured broth at 600 nm using a spectrophotometer (Spectronic 20 Genesys, USA). Flocculating activity were measured daily through the kaolin assay. The relationship between bacterial growth and flocculating activity was analysed using Pearson Product-Moment Correlation in SPSS.

According to the study by Buthelezi et al. (2010), microbial cultured broth can be used directly as bioflocculant source without further extraction. This was in agreement with the findings by Zulkeflee et al. (2016) who had proven that bioflocculants were excreted by bacteria into their surrounding culture broths with flocculating activities exhibited by the cultured broth and the cell-free supernatant (after centrifugation) but was not exhibited by the separated cell. Furthermore, Pathak et al. (2015) reported that both bioflocculant in broth and in its purified form had successfully removed selected metals with similar efficiency. Therefore, the subsequent heavy metal removal assays utilised batch culture broths of the strain that was at its highest flocculating performance, as the bioflocculant source (without extraction).

Pilot Test on Synthetic Heavy Metal Solutions

Synthetic heavy metals solutions of 500 µg/L Cd, Cr and Cu were prepared from their respective stock solutions. About 50 mL of each metal solution was poured into Erlenmeyer flasks and bioflocculant at varying dosage: namely 0.5%, 1%, 2% and 4% (v/v) were pipetted into their respective flasks. A control was also prepared by using sterile broth in place of the cultured broth. The pH of the mixtures were monitored but not controlled. The mixtures were then shaken on an orbital shaker at 150 rpm at 25^{o}C, for 24 hrs (Gomaa, 2012). After 24 hrs the supernatant were collected and filtered through 0.45 µm filter syringes. Clear 4.0 mL filtered samples were then analysed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) for heavy metal concentration. All analyses were done in duplicates.

The percentage removal was calculated based on the formula below.

$$\text{Percentage removal (\%)} = [(A-B) / A] \times 100$$

Where A is the initial concentration of heavy metal and B is the final concentration of heavy metal in the treated solutions.

Heavy Metals Removal from Lake Water Samples

Lake water samples were collected from three sampling points at Cempaka Lake in Bangi, Malaysia and were homogenised for heavy metal analyses. Cempaka Lake is a recreational lake surrounded by industrial, commercial and residential areas. The lake has previously been reported to be polluted by heavy metals due to the various anthropogenic activities surrounding the area (Gasim et al., 2017; Taweel et al., 2013). *In situ* water quality parameters including pH, temperature and turbidity were measured on site. The parameters were compared with the National Lake Water Quality Criteria and Standards (NLWQS) that was developed in 2015 by the National Hydraulic Research Institute of Malaysia (NAHRIM) for Category B lakes i.e. lakes used for recreational purposes that includes secondary body contact such as boating and cruising.

The experiment as described in the pilot test was conducted on the homogenised water samples that were taken from the lake polluted with heavy metals such as Al, Zn, Fe and Cu. These were measured as the highest metals that were present in the water samples. Additionally, the effect of pH on bioflocculant treatment was also investigated by varying the pH of the mixture to pH 2.0, 6.0 and 9.0.

For heavy metal concentrations the levels detected were compared with the NLWQS which also adopted the heavy metal standards of the National Water Quality Standards for Malaysia underlined by the Department of Environment (DOE), Malaysia in 2006.

RESULTS AND DISCUSSION

Bacterial Identification and Characteristics

A mucoid and ropy colony forming bacterial strain with the highest flocculating activity at 97% was chosen and was further characterised and identified. Mucoid forms a colony that has morphological characteristics bearing a slimy and glistening appearance, and is ropy i.e. the ability of colonies to form long strings when touched with a wire inoculating loop. These were reported as good indicators of bioflocculant-producing microorganism (Li et al., 2009). The BBL Crystal Gram-Positive ID System had biochemically identified the strain as *Bacillus subtilis* with a 99% similarity. Figure 1 shows the microscopic image of the Gram-stained strain captured at 4000x magnification using Nikon YS100 light microscope. The image shows rod-shaped Gram-positive bacteria.

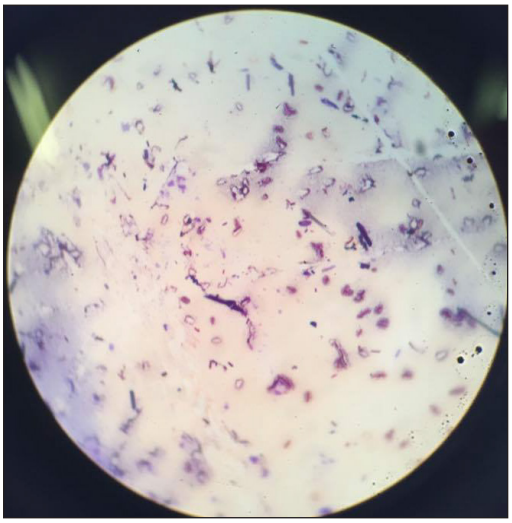

Figure 1. Purple stained rod-shaped bacteria identified as *B. subtilis*

Bioflocculant Production during Growth

Figure 2 shows the flocculating performances of the bioflocculant that was produced during a 72 hrs batch culture of *B. subtilis*. Under controlled condition, the growth slowly increased to 0.098 and hit its maximum growth at 0.904. The growth drastically declined by the third day.

Bioflocculant productions are reflected by the flocculating activities measured during growth (Ugbenyen & Okoh, 2013). Bioflocculant was observed to be produced throughout bacterial growth with a strong positive relationship ($r = 0.81$, $p<0.01$), with high flocculating activity being directly associated with the rapid growth of *B. subtilis.* The maximum flocculating activity measured was 81.96% at the peak of bacterial growth in 48 hrs before similarly declining together with bacterial growth to around 66%. Bioflocculant productions throughout growth could vary with different microorganisms depending on their respective growth rates. Liu et al. (2010) reported that bioflocculant production by *Chryseobacteriumdaeguense* occured during the death phase, while Gong et al. (2008) and Wang et al. (2007) had reported on the production of bioflocculants occurring parallel with logarithmic growth of their respective strains. On the other hand, Su et al. (2012) reported that bioflocculant production by *Arthrobacter* sp. occured parallel with growth but eventually declined by 72 hrs of incubation, which was similar in the case of *B. subtilis* bioflocculant production in this study.

The decline of bacterial growth was mainly due to insufficient provision of nutrient. This was also reflected by the declination of the flocculating activity reading. However, the flocculating activity had maintained above 50% towards the end, indicating that the bioflocculant that was produced had remained in the culture system while some might have been re-uptake by the strain as an alternative food source (Zulkeflee et al., 2016).

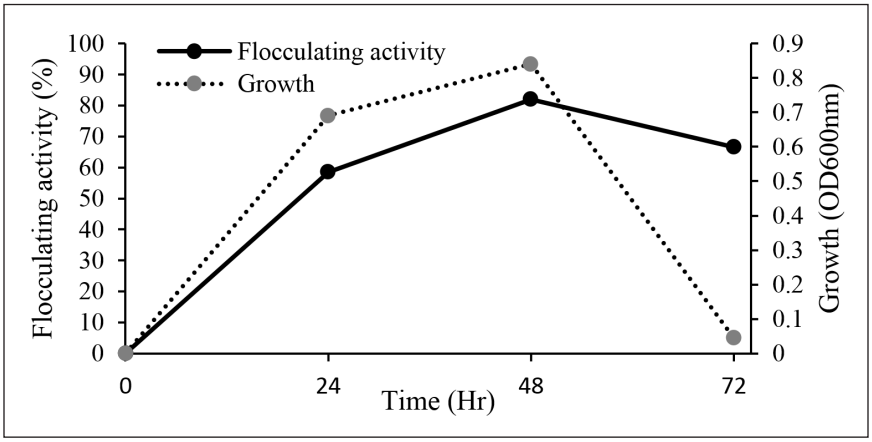

Figure 2. Timeline of bioflocculant production during growth of *B. subtilis*

Pilot Test of Heavy Metals Removal from Synthetic Solutions

A pilot test on the removal of 500 µg/L of Cd, Cr, Cu from synthetic solutions was conducted utilising 48 hrs old cultured broths of *B. subtilis,* which had been tested for its flocculating activity (Figure 3). The removal rate of different metals differed at different dosages of bioflocculant used. The highest removal of Cu (16.3%) was attained at 4.0% (v/v) of bioflocculant dosage while for Cd the highest removal (13.4%) was by using 2.0% (v/v) of bioflocculant dosage. At a higher or lower dosage, the removal of Cd was observed to be lower while for Cu a high removal rate (14.4%) was achieved at 0.5% (v/v) of bioflocculant dosage; however it dropped at the increase of subsequent two-fold bioflocculant dosage before the rate increased at a dosage of 4.0% (v/v). Cr removal was observed to be less than 5% at all applied dosages. The removal rate was also observed to be oddly negative at 1.0% (v/v) bioflocculant dosage for all metals. Nevertheless, all rates were lower compared to the metal bioflocculation reported by Gomaa (2012) and Lin and Harichund (2012).

According to Morillo et al. (2006) different removal rates of various metals by bioflocculants at different dosages were the results of different affinity, charge density and polymer conformation on adsorbed ions of the metal-bioflocculant interaction. This was agreed by Gomaa (2012) who reported on the different optimum dosages for Pb, Zn,

As and Cd removal through bioflocculation by *Pseudomonas aeruginosa*. Hence, it explained the different removal rates that were achieved at different bioflocculant dosage for Cd, Cu and Cr (Figure 3). Effective removal of metals was also reported to favour lower bioflocculant dosages (Das & Santra, 2007). This was in agreement with Lin & Harichund (2012) where removal of Pb, Zn and Hg by bioflocculants from *Paenibacillus* sp. CH11, *Bacillus* sp. CH15 and *Halomonas* sp. increased when the dosages were reduced. However, Cd removal was reported to be highest at high dosages. Fundamentally, the basis of choosing the dosages in this study was in accordance to the low dosage theory; however, it was observed that fluctuations in bioflocculating metals by *B. subtilis* bioflocculant were sensitive even at two-fold dosage differences. This could be due to many other factors that affect bioflocculation performance.

One important factor is the pH, because pH value affects flocculation processes (El-Salam et al., 2017). The initial pH of the treatments were monitored but not controlled and were observed to be in the range of pH 3 to 4. Abdel-Ghani & El-Chaghaby (2014) reviewed that in acidic condition, H^+ competed with metal ions to adsorb onto the biosorption surface of bacterial biomass; the bioflocculant. Therefore, the removal of heavy metals tended to be low as presented in this pilot test because the heavy metal ions might compete with hydrogen ions for biosorption with the bioflocculant.

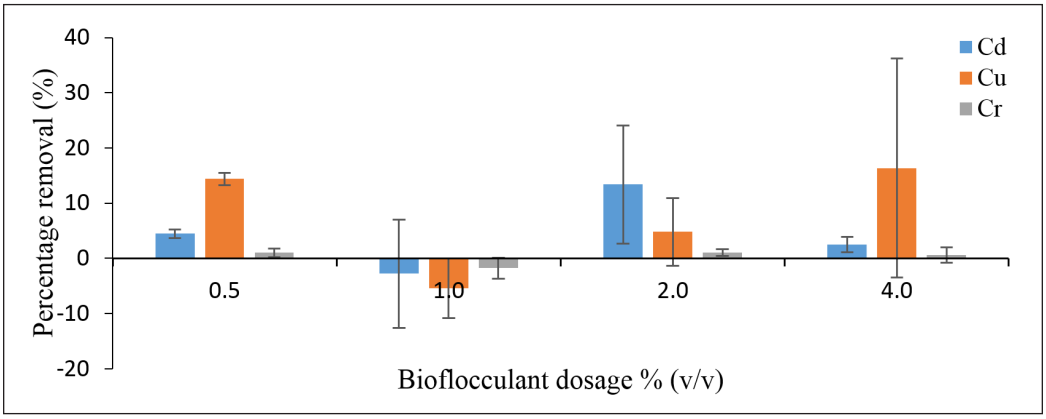

Figure 3. Percentage removal of Cd, Cu, Cr at different dosages of bioflocculant

Heavy Metals Concentrations in the Lake Water Samples

Water samples from Cempaka Lake had an average temperature of 28.9°C, a pH 6.86 and an average turbidity reading of 42.3 NTU. According to the NLWQS all of these parameters were within the range of standards for Category B lakes. Table 1 lists the ten heavy metals that have been measured from the homogenised water

samples from the lake and their comparisons with standard values. The results revealed that Fe was found abundantly in Cempaka Lake with the highest concentration at 999.63±35.46 µg/L, followed by Al and Zn with concentrations of 506.22±25.42 µg/L and 129.22±4.09 µg/L respectively. In contrast, Cr and Ni were found to be absent in the lake with values below detection limit. The concentrations of Cu, Cd, Pb, As and Co were all found to be below the standard limit.

Table 1
Heavy metals concentration in Cempaka Lake and comparison with standard values

Heavy Metals	Concentration (µg/L)	NLWQS (µg/L)
Cd	0.09	2
Cu	3.95	20
Cr	BDL	50
Al	506.22*	100
Zn	129.22	3000
Pb	6.48	50
Ni	BDL	20
As	10.04	100
Co	0.28	50
Fe	996.63	1000

BDL – Below Detection Limit; *exceed the standard value

The concentration of Al in Cempaka Lake had exceeded the standard limit while Fe nearly reached the standard limit, thus both posed as threats to aquatic life as well as to human health. The high levels of Fe detected in the lake had similarly been reported by Gasim et al. (2017) with values exceeding the standard limit. Contradictorily, all other metal concentrations were reported to be below standard limits. In 2011, a similar study conducted by Said et al. (2011) had reported the high occurrences of Zn as compared to other metals. The common trend of detecting high levels of Zn in 2011 and Fe in 2017 proved that there was consistent input of Fe and Zn sources from anthropogenic activities that had increased throughout the years, with the addition of Al that was detected at exceeding levels in 2018.

According to a study by Shuhaimi-Othman et al. (2012), freshwater toxicity of metals on aquatic biota follows the order Cu>Cd>Fe>Mn>Pb>Ni>Zn>Al. Toxicity of Al were reported to be pH dependent where they may become more toxic to aquatic biota in acidic conditions. Thus, it was of interest to investigate the possible removal of heavy metals through bioflocculation.

Removal of Heavy Metals from Lake Water Samples through Bioflocculation

The removal rate of selected heavy metals (Al, Zn, Fe, Cu) by varying the bioflocculant dosages and pH of the lake water homogenised samples are summarised in Table 2. The overall highest percentage of removal was recorded by Zn at 94.3%, followed by Al at 92.9%, Fe at 86.2% and Cu at 68.1%, in an acidic medium of pH 2.0 ($p<0.05$). In an alkaline medium of pH 9.0, most percentages of removal were marked with negative values, indicating that bioflocculation of the selected metals were unfavourable in alkaline conditions. At pH 6, removal of Al, Zn and Fe were maintained to be high around 70-85%. Removal of Cu

on the other hand were low in all conditions. Therefore, the removal of all metals was concluded to favour acidic conditions compared to alkaline conditions ($p<0.05$) with no significant contribution from the varied dosages used ($p>0.05$). Generally, at the optimal pH, bioflocculation of metals differed according to the different types of heavy metals that were being treated (Lin & Harichund, 2012).

According to Gupta and Diwan (2017) H^+ions tends to enhance bridging process that aids in flocculation. This is true based on the results since indication of pH 2.0 and pH 6.0 were more favourable for high bioflocculation of the selected metals due to the higher amount of H^+ ions present ($p<0.05$). At the optimum pH for bioflocculation of metals, efficiency of metal removal is high due to the negative charges saturating the adsorbing surfaces (Pathak et al., 2015). At pH higher than the optimum level, adsorption may be hindered by the formation of hydroxo species of the metals ion that do not bind to the bioflocculant (Lin & Harichund, 2012; Pathak et al., 2015). Furthermore, negatively charged sites and positively charged sites availability fluctuates with pH, with the latter commonly decreased with increasing pH, thus resulting in a lower metal removal at higher pH through bioflocculation (Zhao et al., 2017). Thus, this could explain the negative results obtained at pH 9.

Table 2
Percentage removal of Al, Zn, Fe and Cu from lake water sample at various dosages and pH

Percentage Removal (%)				
Bioflocculant Dosage % (v/v)	**Heavy Metals**	**pH 2.0**	**pH 6.0**	**pH 9.0**
0.5	Al	52.9 ± 44.47	71.7 ± 24.26	-46.6 ± 88.85
	Zn	53.5 ± 43.66	85.6 ± 3.24	1.6 ± 22.90
	Fe	42.0 ± 55.30	71.3 ± 11.90	-24.6 ± 77.70
	Cu	47.8 ± 45.86	47.0 ± 3.71	-6.8 ± 1.94
1.0	Al	92.6 ± 4.17	73.0 ± 19.78	-58.0 ± 94.75
	Zn	94.3 ± 2.42**	84.7 ± 3.74	-16.8 ± 5.72
	Fe	53.7 ± 44.32	67.7 ± 10.86	-58.3 ± 91.50
	Cu	68.1 ± 26.05**	51.4 ± 1.15	-24.2 ± 18.99
2.0	Al	34.0 ± 63.1	51.2 ± 25.63	-29.4 ± 68.23
	Zn	32.4 ± 62.23	69.5 ± 4.70	-23.0 ± 12.05
	Fe	38.4 ± 60.16	64.3 ± 12.50	-19.8 ± 52.76
	Cu	38.8 ± 55.62	46.6 ± 2.61	-49.1 ± 8.37
4.0	Al	92.9 ± 14.05**	32.8 ± 11.61	-6.1 ± 45.71
	Zn	74.0 ± 43.32	60.6 ± 6.74	-11.7 ± 3.67
	Fe	86.2 ± 8.55**	60.6 ± 5.86	-6.8 ± 33.90
	Cu	40.9 ± 43.10	15.4 ± 2.09	-37.6 ± 5.90

**highest percentage removal for each metal ($p<0.05$)

CONCLUSION

Rod-shaped, Gram-positive and mucoid and ropy colony forming bacteria that had been isolated from lake water, and identified as *B. subtilis*, was proven to produce bioflocculants with high flocculating activities. Production of bioflocculant by the strain was found to be positively correlated with growth ($p<0.01$) with the highest flocculating activity measured within 48 hrs of incubation. Possible removal of selected metals (Cd, Cr and Cu) in synthetic wastewater was proven ineffective without pH and bioflocculant dosage manipulations. Further tests on lake water samples that had been polluted with Al, Zn, Fe and Cu showed promising results, with successful removal at 92.9%, 94.3%, 86.2% and 68.1% respectively, in an acidic condition of pH 2 with no significant effects despite the varying dosages that were used ($p>0.05$). Bioflocculant produced by *B. subtilis* was proven to be successful in removing selected metals with promising potential in wastewater treatment applications.

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Review Article

Fisheries Assessment, Gametogenesis and Culture Practice of Local Bivalve: A Review

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ABSTRACT

Hard clams are found to overwhelm the benthic territory from the remote oceans in the marsh region, especially the tropical region. It is a filtered feeder that feeds upon microorganisms, debris and dissolved primal matter as the metabolic vitality hotspot for development. The abundance of food source caused mariculture of hard clam a possible practice in the open sea. Although harvested hard clams through mariculture progressively increase each year, they are still found to be less than oyster and cockle production. Knowledge of gametogenesis is essential for shellfish culture with a specific end goal to resolve the appropriate conditions for breeding. The gametogenesis is ordered into six phases, i.e. resting, early advancement, late advancement, ripe, spawning and spent. Fertilisation between sperm and egg occurs after hard clam spawning by chemical and physical incitement in the environment. Inseminated eggs experience several phases, i.e.; trochopores, D shape hatchlings, umbonal hatchlings and juvenile phase before ending up as mature shellfish. This review is chiefly intended to cover the biology and culture capability of hard clams, especially in Malaysia.

Keywords: Biology, gonad development, hard clams, mariculture, veliger

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INTRODUCTION

Western Coast of Peninsular Malaysia is known for shellfish culture, which consists of *Perna viridis, Paphia undulata, Anadara granosa,* and *Crassostrea* spp. (Poutiers,

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1998; Vakily, 1989). In Sarawak, most of the bivalves (*Modolus* spp., *Pinna* spp., *Placuna* spp., *Polymesoda* spp., *Meretrix* spp.) are gathered from natural environment, with a high demand from the local market (Hamli et al., 2012; Idris et al., 2008; Lovatelli, 1988a).

Among shellfish that are found in natural habitats, *Polymesdoa* spp. (Corbiculidae) and *Meretrix* spp. (Veneridae) are mostly exploited by local communities in the coastal areas of Sarawak as a protein substitute. According to Hamli et al. (2012), these hard clams were traded at seven divisions in Sarawak. Despite a high demand from the market, there is no documentation on hard clam culturing practice from Sarawak. Most fishermen collected it from the mudflat area during the low tides. The coastal area, together with flora and fauna, sustains each other to form a tangible ecosystem. Sarawak itself sustains a large area of lowland of approximately 1.24 million ha or 13% of the total land area (Page, 2011). This area consists of wide areas of mudflats, which provide a proper habitat for hard clams and other bivalve species. Therefore, this area is able to support a large number of fauna consisting of adequate supply of nutrients to help prolong clam heredity.

However, lowland area is vulnerable to the destruction and pollution from rapid urbanisation activities in Sarawak. Pollution from the urban area and factories will damage the natural habitat of clam species. Pollutants commonly derived from sawmills, wood chip and sago factories may destroy the natural habitat of molluscs and other biodiversity (Davy & Graham, 1982). Disturbance of the habitat will alter the nutrient and biotic composition, which eventually affects the survival of hard clams. Moreover, the depleting number of hard clams also may result in overexploitation to meet the market demand, which increases every year.

Morphology

Bivalve is also identified as two symmetrical shells or two valves associated with tendon at the pivot (Spencer, 2002; Webb et al., 1978). Poutiers (1998) described that Veneroida orders had three diverging cardinal teeth belonging to Veneridae and Corbiculidae families. Cardinal teeth are significant in valve movement mechanism as a hinge which is regulated by anterior and posterior adductor muscles (Spencer, 2002). The resting position of adductor muscle will cause the ligament to spring apart the valves and the valves are closed when the adductor muscle turns to a constricted position.

The dorsal area of clams comprises of a ligament which functions as a fixing and is necessitated for valve opening and closing mechanisms. Adjacent to the hinge is a conspicuous bulge known as umbone, which is frequently seen on the dorsal surface and located more in the anterior region (prosogyrate). Most of bivalve umbone is well-developed within a week after fertilisation (Helm et al., 2004). Growths lines can be seen in the outer shell and typically run parallel to the shell margins. Hard clams only have a short siphon,

which is located in the posterior area of the valve since it is not a deep burrow type as compared to the Solenidae family.

These fundamental shell attributes are noteworthy for hard clams distinguishing proof. Different investigations were implemented based on shell features to quantify the development of bivalve and furthermore as an apparatus to contrast between comparative or distinctive species. Several studies have used morphology and morphometric analysis to differentiate the two species of bivalve, such as Pinnidae (Idris et al., 2009; Scheltema 1983). Babaei et al. (2010) also reported on the relationship between shell measurement with the visceral mass and shell weight of *Amiantis umbonella*. Application of morphometric dimension through anatomical character is also important to clarify taxonomic identification of *Corbicula* and its distribution (Araujo et al., 1993).

Growth performance, shell shapes and sizes of hard clam are influenced by environmental factors. Variable physical factors of the environment are known to influence shell morphology and relative proportions of many bivalve species such as latitude (Beukema & Meehan, 1985), depth (Claxton et al., 1998), shore level (Franz, 1993), currents (Fuiman et al., 1999), water turbulence (Hinch & Bailey, 1988), wave exposure (Akester & Martel, 2000), type of bottom (Claxton et al., 1998) and sediment type (Newell & Hidu, 1982). Morphological variation in shells depicts the growth performance of bivalve. Claudi and Mackie (1994) reported the importance of environmental factors for the survival and growth of zebra mussel (*Dresissena polymorpha*). Food availability and population density are also important to determine the shell morphology, shell length and body mass ratio of bivalve (Alunno-Bruscia et al., 2001).

Habitat

Major hard clam species inhabit the estuarine and coastal areas with high nutrient resource and organic matter, which are important to other organisms that inhabit the same ecosystem. Abundance of quality food and nutrient resources are able to support a huge number of organisms that includes hard clam (Bricelj et al., 2017). Moreover, it is also important to influence shell morphology in terms of shell length and body mass ratio of bivalve (Alunno-Bruscia et al., 2001). Furthermore, Kovitvadhi et al. (2006) described that survival rate of freshwater bivalve was affected by diversity of phytoplankton in the habitat.

Formation of bivalve shell can be affected from biotic and abiotic factors in the habitat (Kovitvadhi et al., 2009). Shallow environment can influence the shell characters as detailed by Lajtner et al. (2004), while bivalve population density is connected with sediment form. Most of the coastal areas near the estuarine have sediment characters of sand and mud types which are suitable for *Meretrix* spp. to grow. Other than *Meretrix* spp., *Anadara* spp., which is sometimes found at the same habitat, tends to favour muddy sediments.

They have developed ciliate structures to prevent their branchiae from becoming clogged with fine particles (Broom, 1985; Yoloye, 1975). However, each species may have its own distinct requirements. *Anadara granosa* for example, happens in areas where between 50% to 90% of the substrate is made up of particles under 0.125 mm in measurement (Pathansali, 1966). Some bivalves live on sediment with fine sand over half, mud over 70%, and intermediate granule substance is around 5% to 15% (Baron & Clavier, 1992). Different species recorded can be found on exceptionally coarse coral, non-coral sand, terrigenous sands and medium all around arranged silt with a mean particle size of 0.4 mm (Gibbs, 1978; Narayanan & Sivadas, 1986; Purchon & Purchon, 1981).

Feeding and Nutrient

Bivalves ordinarily utilize the gills optionally inferred part, which are critical in the feeding process. The ciliary tract of gills work on evacuating and arranging the suspended particles from pumping water and this framework is known as suspension filter feeding (Leal, 2002). Food such as phytoplankton that is trapped at the gills will move through the palp, mouth and eventually the digestive system. Generally, feeding on bivalves begins as soon the shell and digestive organ are fully developed. During the larval stage, this organ is still undeveloped, therefore nutrients are fully supplied to bivalve larvae through direct absorption from the surrounding water (Helm et al., 2004). After the shell is fully developed, the food type for bivalve larvae depends on biochemical composition, ingestability and digestibility, which are important for culturing purposes.

Normally, hard clam foods include an assortment of suspended molecules, for example, microorganisms, phytoplankton, waste, dissolved organic matter, amino acid and sugar (Bouillon et al., 2003; Davenport et al., 2000; Nicholas, 1985; Stewart & Bamford, 1975). Information on bivalve nourishment can be resolved through the examination of the bivalve gut content. This sort of examination has been performed by estimating isotopic improvement, consumption inside tissues apart from investigating digestive and absorptive efficiencies (Hawkins & Bayne 1992; Shumway et al., 1987). Generally, bivalves feed on phytoplankton as the primary diet and there is still little data with respect to the detailed food that is consumed by bivalves in nature.

Some bivalves also can be categorized as partially carnivorous since there are a few bivalve species that can ingest zooplankton and benthic organisms (crustacean and bivalve larvae) (Davenport et al., 2000). Seasonal variation on diet type for bivalves occurs especially for algae species. This variation corresponds with the algal bloom periods and it is different for individual algae species. Abundance of phytoplankton in the natural habitat will cause the rapid growth and maximum size of bivalve (Carmichael et al., 2004). However, neither individual nor mixed algal diet produces significant results in *Meretrix meretrix* larvae survival as

reported by Tang et al. (2006). Although the phytoplankton is the most important food source, the availability of detritus during the scarcity of phytoplankton at certain periods can help to provide energy for metabolic activity in bivalves. However, only certain detritus can be ingested by bivalves and it depends on the particle size. Venerids and others molluscs comprised appropriate gill filaments (eulamellibranch) to ingest extensive molecules up to 950 µm, and they have great maintenance productivity for molecules measured as little as 1 µm (Jĝrgensen, 1990; Mikulich & Tsikhon-Lukanina, 1981).

Dissolved organic matter in wetlands and oceans is vast, which also serves as the source of nutrient supplement for benthic organisms including hard clams, especially during the scarcity of the essential nutrient (Bouillon et al., 2003). High organic matter is also contributed from fish by-products, which is commonly found near the fish farming areas. Bivalves can transport dissolved organic matter in the form of sugars and free amino acid to the gills and absorb it for metabolic processes (Stewart & Bamford, 1975). The study indicated that *Mytilus edulis* can uptake 94% of natural amino acid and have as low as 10% of it from the ambient seawater (Hawkins & Bayne, 1992; Manahan et al., 1982).

Culture Production

The earliest culturing practice for bivalves was traced back during the Roman civilisation. Oyster was cultured at that time due to the high market demand since it was utilised as food and the ability to produce pearls for jewellery. Bivalves or molluscs from the marine environment received more attention because of their aesthetic and gastronomic appeal (Subba Rao, 1993). There are many types of edible bivalves around the world, but currently only oysters (*Crassostrea*, *Saccostrea*, *Ostrea*), mussels (*Mytilus*, *Perna*), scallops (*Amusium, Pecten*, *Chlamys*, *Aequipecten*), cockle (*Anadara*) and clams (*Ruditapes philipinarum*, *Tapes philippinarum*, *Meretrix lusoria, Mercenaria mercenaria*) are being applied for mariculture practices in the Asian region (Lovatelli, 1988a).

Shellfish Production. Most countries in the tropical area are culturing bivalve intensively to support the local demand. From 1991 until 2005, production of bivalves indicated an increasing pattern with 6.3 million tonnes in 1991, 9 million tonnes in 1995, 13 million tonnes in 2000 and 23.6 million tonnes in 2005 (Figure 1). The increasing pattern is due to the rapid growth of aquaculture and bivalve production in China (Pawiro, 2010). While in the year 2005, 87% of bivalve landing is a result of culture activities (Globefish-FAO, 2007). Therefore, a significant difference between cultured and wild-captured bivalves was observed from 1991 to 2005. The production was indicating wild stocks were incapable of supporting market demand in which the scarcity in production was supported by culturing practices. This pattern certainly will continue to increase for a few years ahead to coincide with the increasing human

population around the world. Reliance on the wild stock to meet this rising demand definitely will cause extinction for certain bivalve species in which the only solution is through the culture practice.

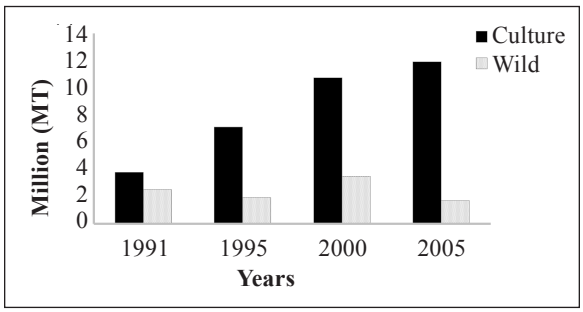

Figure 1. Comparison of cultured and wild captured bivalve for 1991, 1995, 2000 and 2005. Adapted from Globefish-FAO (2007) and Helm et al. (2004)

There are four major marine cultured bivalves currently practised in a few continents (Table 1). Asia has turned into the primary area that adds to the world aquaculture products, including bivalves (Oyster, clam, scallop). Clam is the most elevated bivalve production with 39% yield (Figure 2), which is half of the bivalve world production. Clam production as of late has expanded to 24% of generation because of trade from scallop to clam culture. High mortality in scallop culture in China caused this change due to the high survival rate of clams in contrast with that of scallops (Guo et al., 1999). Meanwhile, different species contributed between 10% to 15% of the world production.

However, Malaysian bivalve generation as opposed to the world bivalve production shows that oyster is the species to contribute the least in bivalve production either from wild or aquaculture sources. Significant

Table 1
Major marine cultured bivalve in the world (Garibaldi, 1996)

Species	Continent
Oyster	Africa, America, Asia, Europe and Oceania
Mussels	Africa, America, Asia, Europe and Oceania
Scallop	America, Asia, Europe and Oceania
Clam	Africa, America, Asia Europe

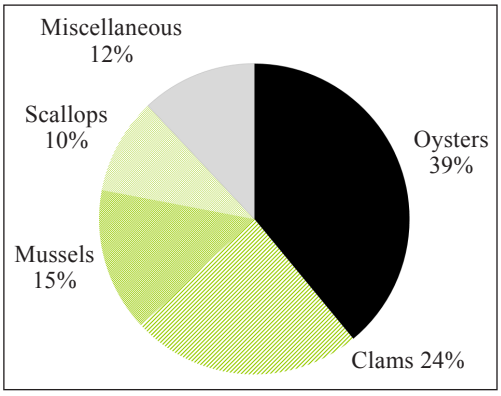

Figure 2. World production of cultured bivalve in year 1999 (Food and Agriculture Organization [FAO], 2001)

mollusc production in Malaysia originates from cockle, which is in conflict to bivalve world creation (Figure 3). Cockle contributed to 67% of Malaysian bivalve production that is followed by clams (28%) and mussels (4%). This examination depends on 12-year allotment which demonstrated Malaysia is still not utilizing its resources at the ideal level to expand the bivalve production through mariculture. However, bivalve production in Malaysia will keep on growing in perspective in the way that Thailand and Singapore are the premier merchants of the bivalve from Malaysia.

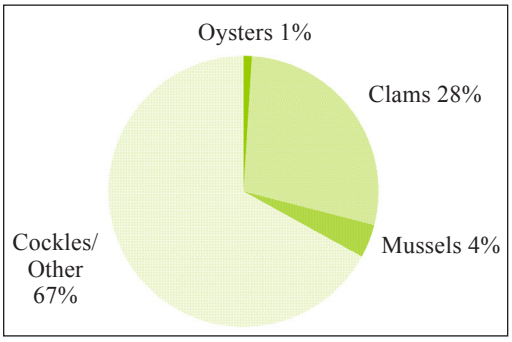

Figure 3. Malaysian bivalve production in year 2011 (Department of Fisheries Malaysia [DOF], 2013)

Hard clam is the suitable animal for mariculture since this animal is akin to herbivores in filtering most of the phytoplankton from the surrounding water and this acquired less concern (Nicholas, 1985). The introduction of hard clam varieties in mariculture is necessary since current commercial production is only dependent on a few bivalve species, while other species are only harvested for normal daily consumption. Fishermen favoured harvesting bivalves from the wild due to some obstacles to cultivate the mollusc. Furthermore, harvesting from wild stock is less expensive as compared to culture which brings with it the need for seed production, nursing, grow out, planting and harvesting (Edwards, 2000; Quayle & Newkirk, 1989). Culture practice for certain hard clams will become non-competitive especially if the species of interest is found abundantly in nature, for instance *Polymesoda* spp. (Hamli et al., 2012).

Semi-culture practice is widely used for hard clam culture since it can promise high clam production during harvesting, resulting from the high density of clam seed planting. However, there are restraints in this kind of culture, especially when related to the seed collection. Seed collection from the wild stock usually depends directly on the amount of seed produced in nature and if seed production is insufficient, it will definitely affect the number of clams harvested (Helm et al., 2004). Breeding season on certain species also affects the seed source for annual production. An alternative way to get enough supply of seeds without depending directly on season changes is by artificial propagation of the clam seed or spat.

Other than seed supply, water quality is an important element to ensure the success of hard clam culture. Semi-culture practice has less intention for water quality maintenance while planting seed involves the natural habitat of the bivalve. The only concern in hatchery culture, especially seed production is water quality. Water quality variables in terms of salinity, temperature

and dissolved oxygen are important for broodstock breeding and to ensure the survival of the larvae after fertilization. Water salinity is one of the components for clams that live in marine habitats due to its effect on the osmotic physiology of the organism (MacLachlan & Erasmus, 1974). Low salinity levels will change the spawn activity of bivalve species (Baba et al., 1999). Marine bivalves only tolerate high concentration of salinity, for example *Perna viridis* can tolerate low salinity of 16 ppt whereas other bivalves from estuarine or brackish sources can tolerate a broader level of salinity (Sundram & Syed Shafee, 1989). At the tropical areas, the native bivalves can tolerate high temperatures. For that, water temperature in hatchery cultures should not be too low since it can affect larval growth and survival during culture. When water temperature is high, it will lower the dissolved oxygen in the water by increasing the organism respiration. Prolonged circumstance will cause the bivalve larvae stress and eventually die (Laing & Spencer, 2006). Water quality in hard clam culture is a crucial aspect that should be monitored during the rearing period.

Gonad Development

Hard clam sex can be isolated into male and female (dioecious) and a portion of the animal varieties can form into two genders in a single individual (hermaphrodite). Venerids especially *Meretrix* spp. is dioecious as examined from different studies from the Asia site (Chung, 2007; Durve, 1964; Jayabal & Kalyani, 1987). Notwithstanding, Chu and Kumar (2008) had discovered that only 6% of the *Meretrix lyrata* population comprised a hermaphrodite kind. Hard clams need reasonable states of water saltiness, temperature and satisfactory supplement for gonad development and spawning. Sperm that is secreted into the natural habitat will stimulate female clams to spawn. Common spawns happen at a specific temperature level extending from 4°C to 37°C contingent upon mollusc species and areas (Belda & Del Norte, 1988; Philippart et al., 2003). Tropical region normally consists of mature hard clam species throughout the year. Therefore, spawning activities also occur continuously throughout the year.

Eggs and sperm production (gametogenesis) occur in ciliated ducts known as gonad. The gonad will undergo several stages of development before clams mature and are ready for spawning. The classification of the gonad stages of bivalve is shown in Table 2.

Various methods can be applied to determine the gonadal stages of bivalve and the most commonly used methods are through histology and condition index. Histological method is a technique that needs the sacrifice of the animal in order to determine the gonad stages (Larson et al., 1971; Pronkere et al., 2008). Small amounts of gonad from the bivalve are extracted before undergoing the smearing process. Then, obvious stages of male and female gonads can be determined through microscope observation (Figures 4 and 5).

Table 2
Classification stages of bivalve gonad (Seed, 1975, 1976; Wilson & Seed, 1974)

Stage	Description
Resting	On active stage where there is no sexual activity in process. Gonad indicating genital canals in a collapsed state, lipid and glycogen filled the connective tissue. At this stage, male and female are difficult to identify.
Development (Early)	Gametogenesis begins to occur with little cluster of the germinal cells found spread out to the connective tissue. At the follicles, wall oogonia and spermatogonia are developed. Identification between male and female is still difficult.
Development (Late)	Most of the mantle occupied by male or female follicles respectively. Masses of spermatocytes and spermatids fill the follicles while spermatozoa are scattered among the larger cells in males. While female oocytes have started to accumulate yolk and have grown considerably. Some of the larger oocytes are still attached to the follicular epithelium. Some of the gametes start to ripe and gonad mass is increased by half in the mature condition.
Mature	Gonad fully ripes with follicle filled by spermatozoa with a visible tail. Spermatozoa assemble in lamella, congregating in the centre of the lumen. Only few residual spermatocytes and spermatids may be present. However, in females, the maximum size of oocytes assemble together in the follicles. With the increasing size of follicle, connective tissue is covered. Glycogen and lipid are absent in the connective tissue.
Spawning	Gamete starts to have active secretion. Large numbers of ripe oocytes are still present in the follicles. Residual oocytes tend to be rounded as the reduced number of compact follicles. Sperm number at lamellae is reduced to absent.
Spawned	After final spawning, the follicles begin to collapse and degenerate. Smaller numbers of unspent gametes are rapidly broken down by amoebocytes or undergoing cytolysis and the animal again enters the resting stage.

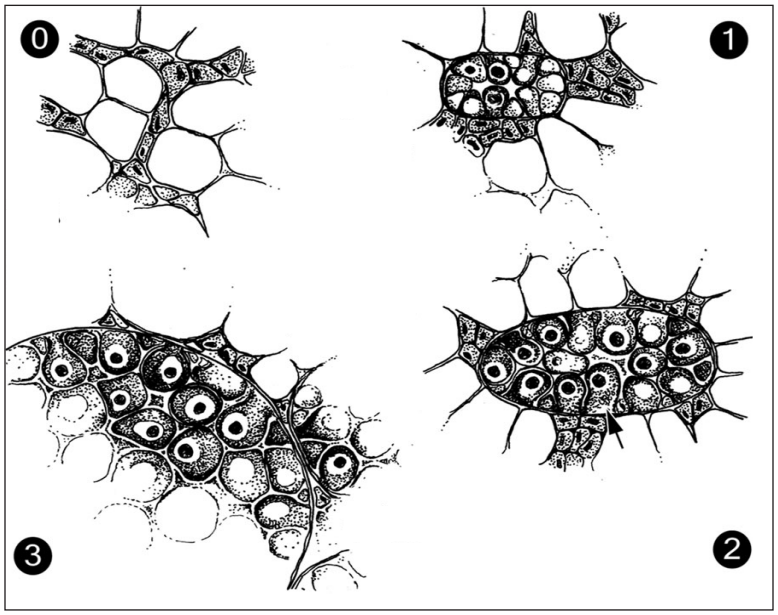

Figure 4. Development stage of ovary. Stage 0: rest, Stage 1: early development, Stage 2: late development, Stage 3: Mature (Duinker et al., 2008)

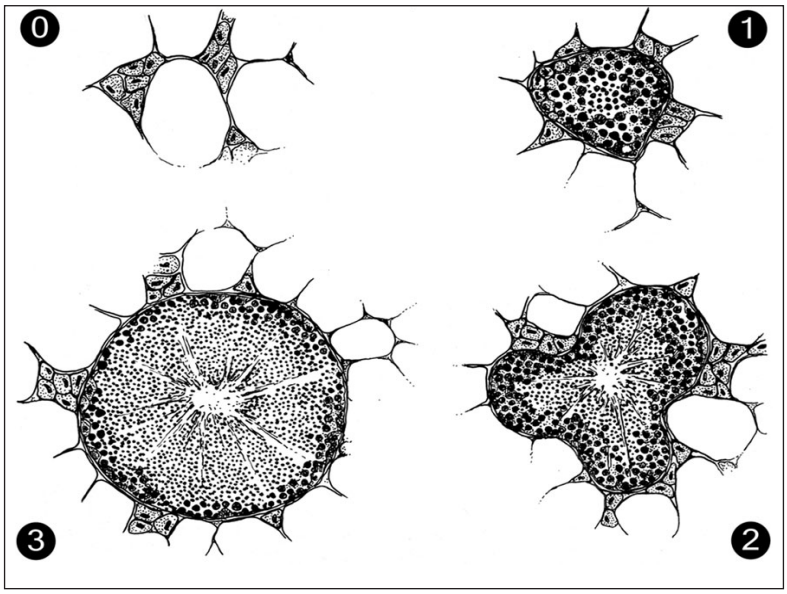

Figure 5. Development stage of testis. Stage 0: rest, Stage 1: early development, Stage 2: late development, Stage 3: Mature (Duinker et al., 2008)

The technique is accurate to identify the gonad stage and sex. However, condition index manipulates the quantitative value on weight of dried soft tissues that is divided with shell weight and multiplied by 100 percent (Davenport & Chen, 1987). It is the cheapest and fastest way to determine bivalve's gonad development compared to histology technique (Austin et al., 1993; Schumacker et al., 1998; Scott & Lawrence, 1982).

Embryonic and Larval Development

During matured phase, hard clam tissue has developed to be more muscular since increasing in mass and size of gametes in both female and male gonads. Increasing of meat weight in bivalves usually is the best time for harvesting because meat productivity is ideal for consumption (Lucas & Beninger, 1985). Physico-chemical changes in the seawater embark the releasing of hard clam sperm into the environment and inducing the female to spawn as well. Secretion of sperm and egg can be identified through the development of a pale cream (Sreenivasan & Rao, 1991) or yellow (Dharmaraj et al., 2004) colour in the water column.

Normal reproducing happens to correspond to the ambient conditions of temperature and salinity in the seawater. This causes some bivalve species of only spawning several times a year, while other species from tropical regions are able to spawn throughout the year. Artificial breeding is the best way for seed production without depending on the seasonal changes. Prior to that, cultured spat can be transplanted to the natural habitat for grow out anytime. In the ongoing years, a few

studies led to the trigger of clam breeding for a hatchery reason (Table 3). There are two different ways to induce bivalve breeding, for example, chemical incitement by means of a serotonin infusion (Neo et al., 2011) and physical inducement by means of thermal stun, salinity shock and air-drying (Chu & Kumar, 2008).

Table 3
Clams species that have been propagated through artificial breeding

Species	Spawning stimulus	Larval duration	Sources
Tridacna squamosa	Serotinin	2–18 days	(Neo et al., 2011)
Ruditapes philippinarium	Air dried and Thermal shock	20 hours–13 days	(Hur et al., 2005)
Mactra veneriformis	Air dried and Thermal shock	18 hours–14 days	(Hur et al., 2005)
Cyclina sinensis	Air dried and Thermal shock	2 days–17 days	(Hur et al., 2005)
Meretrix lusoria	Air dried and Thermal shock	18 hours–7 days	(Hur et al., 2005)
Potamocorbula amurensis	Thermal shock	2–27 days	(Nicolini & Penry, 2000)
Meretrix casta	Thermal shock	2–9 days	(Sreenivasan & Rao, 1991)

The fertilised egg will form into planktonic larvae, which comprised a few phases (Figure 6). First phase is named as trochophores, trailed by veliger larva phase (D larva phase or prodissoconch I phase, prodissoconch II phase), postlarvae phase, which experience transformation known as juvenile or spat (Reverol et al., 2004; Schejter et al., 2010; Suquet et al., 2013).

Trochophores stage is cell division that occurs within an hour after fertilisation success (Suquet et al., 2013). Veliger larvae stage occurs within 48 hours, in which the shells start to develop and eventually wrap the body. During this period, the essential organ system that includes the swimming organ is formed (Garrison & Morgan, 1999). A bivalve larvae swimming organ known as velum is ciliated and also functions in food collecting. Morphology of the larvae will change with straight at the hinge area to form a 'D' shape which is called as the D larvae stage (prodissoconch I stage). This stage prolongs until a week before larvae enter the prodissoconch II stage. Umbone starts to develop in prodissoconch II stage and becomes prominent as the larvae grow larger (Chanley & Andrews, 1971). Hur et al. (2005) divided the prodissoconch II stage into two groups, which are early umbonal veliger and late umbonal veliger. Larvae reach the mature stage after the foot and gill are prominent. Larvae that begin the sedentary benthic are now in spat stage or juvenile. This stage is appropriate for seed collection and transplant into selected natural habitat for culture practice.

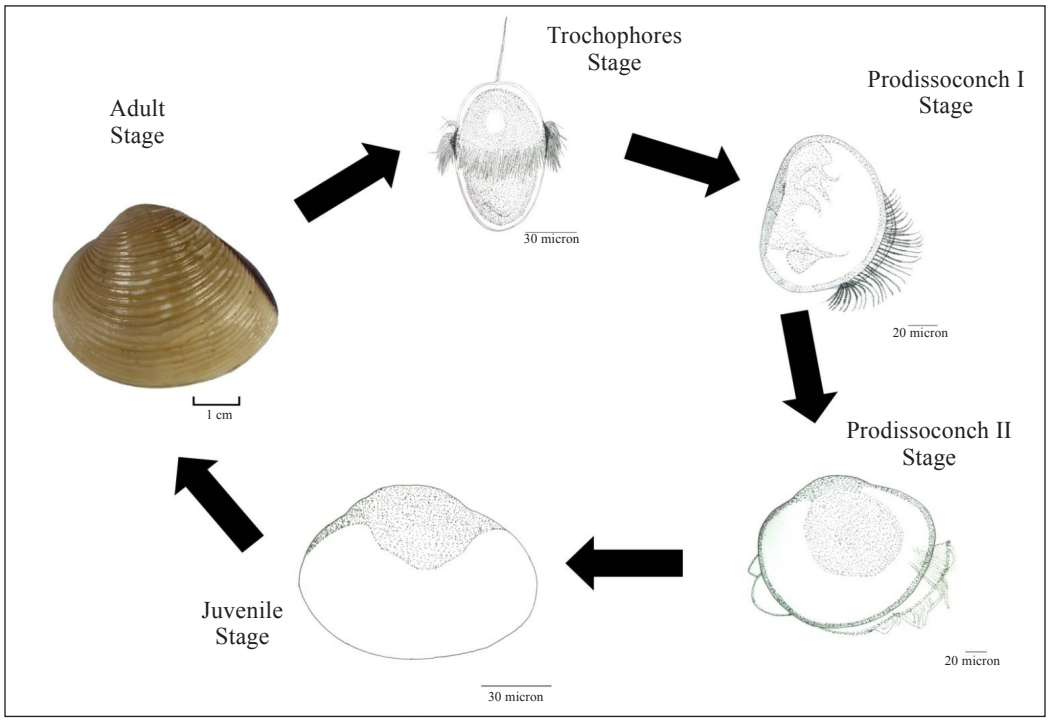

Figure 6. Bivalve larval development stages after Reverol et al. (2004) and da Costa et al. (2008)

Culture Technique

Simple innovation and less labour obtained for hard clam culture is an imperative perspective to urge individuals to include in bivalve cultivating. Advanced technology, improvised methods and effectiveness in hard clam culture are the main keys needed to overcome the rise in market demand for this sort of fisheries product. Technology in aquaculture is also important in dealing with physical and mechanical aspects to deal with water, cultivated organisms and nursery (Ernst et al., 2000; Lee et al., 2000; Papandroulakis et al., 2002; Summerfelt et al., 2000). Moreover, the reliable characteristic for instance, inexpensive seed supply and fast growing species are also important in hard clam culture (Davy & Graham, 1982). Furthermore, hard clam provides a protein source for human consumption and serves as decorative accessories in ornamental industry, which gives a high value to the hard clam (Baylon, 1990).

Selecting an appropriate site for clam cultivation is an important aspect affecting growth and survival for the clam. Area of culturing must be sheltered from any extreme weather of strong waves that can wash out the sown spat. In addition, good quality of physical-chemical condition, especially temperature and salinity are also important. At the tropical regions, high temperatures throughout the year causes the abundance of phytoplankton, which is a food source for filter feeders. Culture

areas must not be near the industrial areas to prevent ingestion of industrial waste, sewage and other pollutants (Fujiya, 1970).

Currently, application of semi-culture practices for bivalve farming is worldwide. Semi-culture practices are involved in seed collection from the wild nature and transplanting at one area for proper growth. Low cost and easy harvesting are the crucial parts of this technique that are able to be applied in every country. This culturing method is practised in the United States, Portugal, China, Taiwan, Japan, Korea, Vietnam, Philippines, Sri Lanka, Malaysia, Indonesia and Thailand (Angell, 1986; Baylon, 1990; Chu & Kumar, 2008; Gosling, 2003; Lovatelli, 1988a; Whetstone et al., 2005).

Growing bivalve spat at the culture side has been done for a few species such as clam, oyster, mussel, and scallop (FAO, 2001). Different culture techniques are applied due to the various bivalve species behaviour in the wild habitat. Appropriate techniques applied will ensure the survival of the bivalve through the cultivation period. Moreover, the practice technique enables the non-native or exotic bivalve to be introduced in the particular area. The introduced species are found to be more in areas that do not have native bivalve to be cultured (Davy & Graham, 1982). There are two fundamental procedures presently used in bivalve culture, for example, bottom and off-bottom culture. Many modifications have been made based on these two techniques to lower the cost, adjusted to location condition and authority regulation.

Off-bottom Culture. Developing bivalve spat over the substrate or sand is known as off-bottom culture and this is suitable for oysters and mussels. Cultured bivalve by this technique is generally kept in the crate, sack, net and enclosure (Gervis & Sims, 1992; Walton et al., 2013). This method is practiced either in the intertidal zone or at the ocean. With advanced technology, adapted to the geographical area and local conditions, man had invented culture gear that expands the off-bottom culture into a new level. Presently, there are four strategies in view of off-bottom culture, for instance, raft, rack, long line and trestle frameworks (Figure 7).

Rack and trestle systems are only applicable in the intertidal area as both methods need stakes that are fixed to the seabed. However, raft and longline systems are applicable at the open sea due to the floating feature. Despite topographical adaptation, off-bottom culture gives points of interest to the bivalve culture in terms of development, survival and attention (Walton et al., 2013). Bivalves that are cultured close to the water surface will have fast development because of the wealth of phytoplankton (Mackey et al., 2002; Paerl, 1988). Cultured bivalves are also free from predators and burial due to distance from the seabed, which will increase the survival rate during culture. Furthermore, in terms of nursing, fouling organisms are easily controlled and removed from the shell. Notwithstanding the favourable circumstances, off-bottom has hindrance in terms of cost when considering the labour and gear equipment (Maeda-Martinez et al., 2000).

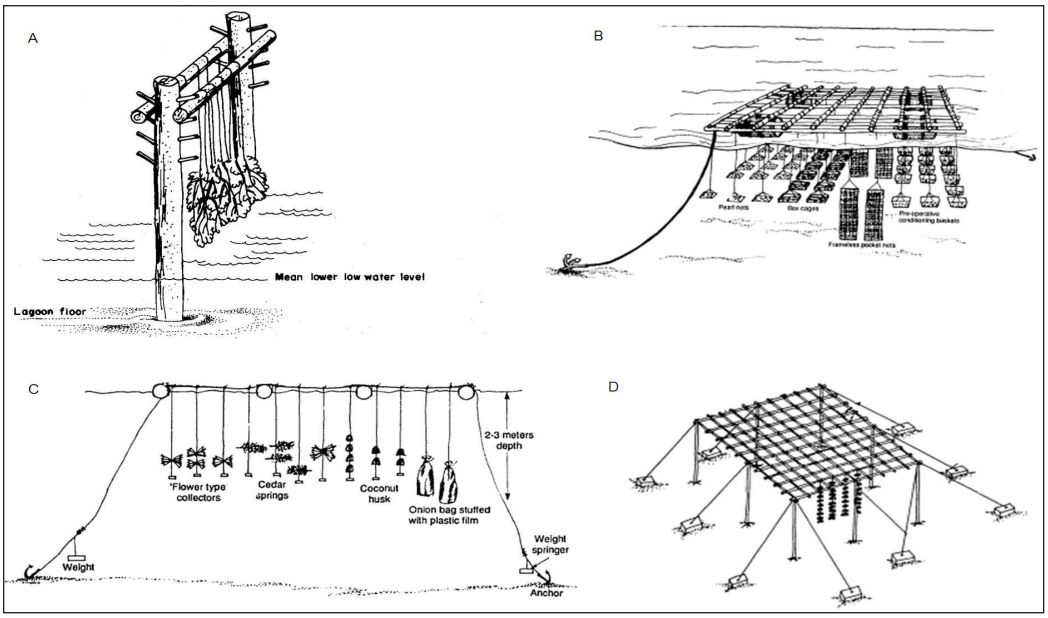

Figure 7. Types of gear used for off-bottom culture. (A) Rack system, (B) Raft system, (C) Longline system, (D) Trestle system (Gervis & Sims, 1992; Lovatelli, 1988b)

Bottom Culture. Bottom culture is alluding to bivalve seed scattered uninhibitedly onto the seabed (Kleinman et al., 1996). Certain bivalve species, for example, oysters will probably join the stone that is present on the seabed. Besides, seabed can serve as the support for a few simple equipment, for example, cage, bottom bag, frame, and rocks for bottom culture (Figure 8). Simple equipment is adjusted to improve the management, nursing and harvesting process.

This culture technique is broadly used in bivalve culture, generally for hard clams which need a substrate for interment behaviour (Table 4). In general, bottom

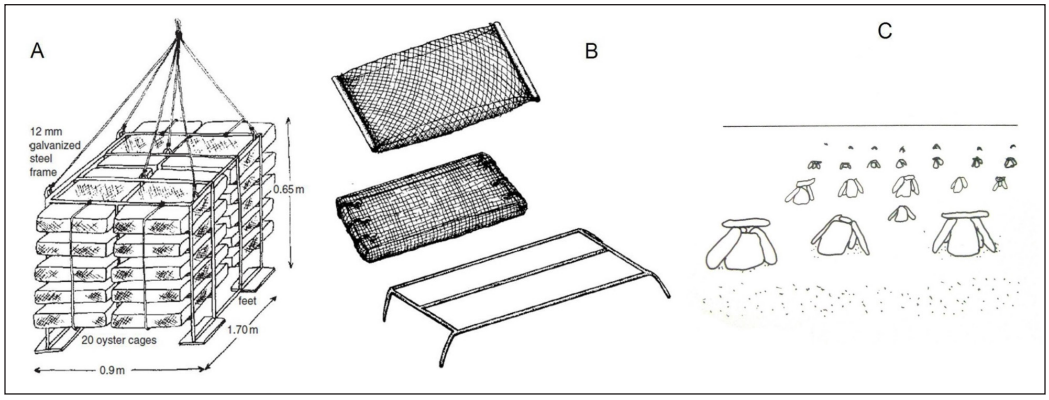

Figure 8. Types of gear used for on-bottom culture. (A) Cage system, (B) bottom bag system, (C) rock system (Cai & Li, 1990; Hardy, 2006; Toba, 2002)

culture is applied in the intertidal area with the intention of easier harvesting process and nursing.

Although bottom culture only uses minor equipment, it benefits the culturist in terms of cost and less work being required for the underlying setup and nursery. Besides that, bottom culture can change the biological system structure by expanding the biodiversity in the natural surroundings (Ysebaert et al., 2009). This demonstrates the execution of bottom culture can enhance the organic accessibility in the sediment. Furthermore, exposed bivalves to the residue will cause bivalves, particularly spat, being powerless against predators like crabs and birds (Toba, 2002). This would therefore result in a low survival rate of the cultured bivalve.

Table 4
Common bivalve species culture by bottom culture system

Common name	Species	Location	References
Mussel	*Mytulis edulis*	Holland, Denmark	(Baylon, 1990; Dolmer et al., 2012)
	Perna viridis	India	(Kripa & Surendranath, 2007)
Cockle	*Anadara granosa*	Malaysia	(Davy & Graham, 1982)
Clam	*Paphia malabarica*	India	(Appukuttan et al., 1993;
Clam	*Mercenaria mercenaria*	United State	Whetstone et al., 2005;
Clam	*Ruditapes philippinarum*	France, Ireland	Gosling, 2003)
Oyster	*Crassostrea edulis*	United State, France	(Robert et al., 1991; Toba, 2002)
Scallop	*Patinopecten yessoensis*	Japan	(Hardy, 2006)

CONCLUSION

Hard clams are shellfish that have a huge part in marine protein creation and because of that, scientists have created numerous strategies to propagate the clams in a small or huge scale. Culture of hard clams, particularly *Meretrix* spp. begins from brood stock conditioning, breeding, larvae rearing, transplanting and lastly harvesting. Prior to the culture application, it is fundamental to comprehend the factors that control the natural limits in the hard clam, for instance, a high measure of meat that is available during the development stage in gametogenesis, which is a reasonable period for harvesting. In this manner, hard clams can be exposed as the substitute protein source of marine that gives profitable value to the country.

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Short Communication

Prevalence, Risk Factors and Transmission of Nervous Necrosis Virus in A Hatchery Producing Hybrid Grouper (*Epinephelus lanceolatus* × *Epinephelus fuscoguttatus*) Fry

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ABSTRACT

This study investigates the prevalence, risk factors, and transmission of nervous necrosis virus (NNV) in a hatchery producing hybrid grouper (*Epinephelus lanceolatus* × *Epinephelus fuscoguttatus*) fry. The eggs and sperm of giant grouper (GG) and tiger groupers (TG) that were collected for breeding purposes within the 12-month study period were sampled to detect NNV. At the same time, three breeding attempts of different NNV status of broodstocks, which were NNV-positive GG × NNV-positive TG, NNV-positive GG × NNV-negative TG and NNV-negative GG × NNV-negative TG were conducted. The produced hybrid grouper (HG) fry was then sampled at 5, 10, 20, 30, 40, 60, 90, and 120 days post-hatched to detect the presence of NNV. The fresh fish, live feed, and commercial fish pellet that were used to feed the broodstocks or HG fry throughout the study period were also sampled for NNV detection. The water's physico-chemical parameters during each sampling were determined. The results revealed that the broodstocks had a low

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prevalence to NNV. However, when at least one of the broodstocks was NNV-positive, all batches of the fry were NNV-positive at high prevalence. There were consistent associations of ammonia and iron with the presence of NNV in both broodstocks and fry. Phylogenetic tree indicates the possible horizontal and vertical transmissions of NNV in the hatchery culture system. Understandings the epidemiology of NNV in a real hatchery condition can provide significant information for control and prevention of the disease.

Keywords: Hatchery, prevalence, risk factors, transmission, viral nervous necrosis

INTRODUCTION

The marine aquaculture industry is rapidly expanding in Malaysia. As a major commodity for protein supply, the total production from marine aquaculture (excluding seaweeds) was ~98,050 metric tonnes (MT), with an estimated wholesale value of USD500 million in 2016 alone (Annual Fisheries Statistic [AFS], 2016). The productions of groupers (*Epinephelus* spp.), snappers (*Lutjanus* spp.) and Asian seabass (*Lates calcarifer*) were 6,167 MT, 16,020 MT and 15,026 MT, with estimated wholesale values of USD 55 million, USD 97 million and USD 63 million, respectively, dominating the marine aquaculture production in Malaysia (AFS, 2016). It is expected that the production of these highly valuable marine fishes will increase in the future due to the intensification and commercialization of the industry (AFS, 2016).

The hybrid grouper (♂ giant grouper *E. lanceolatus* × ♀ tiger grouper *E. fuscoguttatus*) was first introduced in Malaysia by Ch'ng and Senoo (2008). Now, it is a favourite among marine fish farmers in Malaysia for their fast growth rate compared to the commonly cultured grouper species (Sufian & Nik-Haiha, 2015). Furthermore, it possesses better resistant towards disease, temperature, and salinity, making it an important target species in aquaculture (Senoo, 2010).

Betanodavirus, the nervous necrosis virus (NNV), causes a disease known as viral nervous necrosis (VNN). It is currently classified into four major genotypes based on the T4 variable region of RNA2: the striped jack nervous necrosis virus (SJNNV), the tiger puffer nervous necrosis virus (TPNNV), the barfin flounder nervous necrosis virus (BFNNV), and the red grouper nervous necrosis virus (RGNNV) (Nishizawa et al., 1997). NNV infects more than 70 species of marine and freshwater fish globally (Doan et al., 2017). The disease is characterised by extensive neuropathy and retinopathy of the brain and eye of affected fish larvae and fry. High mortality rate can reach 100% is usually observed in larvae and fry, but larger fish could also be infected (Kua et al., 2013). Mortality usually occurs following abnormal swimming behaviours, especially at juvenile stages (Nakai & Mori, 2016). In Malaysia, NNV has been detected in marine cultured Asian seabass, humpback grouper (*Cromileptis altivelis*), brown marbled grouper (*E. fuscoguttatus*), golden pompano (*Trichinotus blochii*), and

cobia (*Rachycentron canadum*) (Abdullah et al., 2017; Kua et al., 2013; Manin & Ransangan, 2011; Rangsangan et al., 2011; Ransangan & Manin, 2010). This study intends to determine the prevalence, risk factors, and transmission of NNV in a hatchery producing hybrid grouper (HG) fry. It was designed without any intervention of the hatchery management and practices to allow us to understand the epidemiology of NNV in real hatchery conditions.

MATERIALS AND METHODS

Sampling Site

The study was conducted in a marine fish hatchery located in Besut, Terengganu, Malaysia. The broodstocks of the male giant grouper (GG) (*E. lanceolatus*) and female tiger grouper (TG) (*E. fuscoguttatus*) that were used in this study were reared in cylindrical concrete and rectangular fiber tanks. The broodstocks were fed daily with commercial fish pellet and fresh fish.

Samples Collection from Broodstock and Fresh Fish

Sampling was conducted between March 2016 and April 2017. During the study period, breeding was conducted at monthly intervals, as previously described (Ch'ng & Senoo, 2008; Sufian & Nik-Haiha, 2015). Briefly, the eggs and sperm were stripped from matured TG and GG broodstocks (Glamuzina et al., 1998). In each striping and hormone injection process, the broodstocks were anesthetized using MS-222 (Sigma-Aldrich, Kuala Lumpur, Malaysia) at a rate of 0.1 ml/L of water. Strippings were carried out as soon after the ovulation process occurred, within 6 to 12 hours following the injection of human chorionic gonadotropin hormone (Pregnyl®, Baxter Oncology, Halle, Germany), at a dose of 1000 IU/kg for TG. The total length and body weight of individual broodstocks were recorded before the hormone injection. Prior to the start of breeding, the eggs and sperm of TG and GG broodstocks were sampled to detect the presence of NNV. Two to three broodstocks of GG and TG were used monthly for breeding purpose (Table 1). At the same

Table 1
The status of NNV in grouper's broodstocks throughout the study period

Month and Year	GG♂		TG♀		GG♂	TG♀	GG♂	TG♀
	Length (cm)	Weight (kg)	Length (cm)	Weight (kg)	VNN Status	VNN Status	% Positive	% Positive
March 2016	155.1	80.58	64.0	5.88	-	-		
	160.3	90.64	52.0	3.09	-	-	0	0
	167.0	92.78	65.3	7.33	-	-		
April 2016	150.2	75.95	47.5	2.50	-	-		
			48.8	2.13		-	50	33.3
	146.2	85.84	46.9	2.07	+	+		

Table 1 *(continue)*

Month and Year	GG♂		TG♀		GG♂	TG♀	GG♂	TG♀
	Length (cm)	Weight (kg)	Length (cm)	Weight (kg)	VNN Status	VNN Status	% Positive	% Positive
July 2016	145.6	70.35	51.5	3.46	-	-	0	0
	150.8	80.78	52.5	3.50	-	-		
August 2016	151.0	80.68	51.5	3.80	-	-	0	0
	145.0	70.88	68.9	6.05	-	-		
September 2016	134.6	80.74	54.0	4.60	-	-	0	0
	167.8	93.32	52.4	3.10	-	-		
October 2016	150.3	80.02	55.4	6.24	-	-	0	0
	146.6	85.12	47.9	4.18	-	-		
November 2016	150.8	80.31	53.5	2.89	-	-		
			67.5	5.66		-	0	0
	140.5	80.2	55.5	4.77	-	-		
December 2016	150.1	80.03	73.5	5.54	-	-		
	140.7	65.51	54.5	3.81	-	-	0	0
	155.7	80.41	75.6	5.17	-	-		
January 2017	150.6	80.32	53.5	5.00	-	-		
	150.8	75.45	52.5	3.47	-	-	0	0
	140.6	66.77	50.0	5.11	-	-		
February 2017	140.8	78.00	47.0	4.70	-	-		
			66.5	5.50		-	0	0
	141.8	78.25	62.8	5.10	-	-		
March 2017	155.4	80.52	60.8	7.00	-	-		
	150.6	80.23	56.9	6.50	-	-	0	0
	155.5	81.89	50.3	4.90	-	-		
			54.1	6.40		-		
April 2017	141.4	66.54	60.9	5.60	-	-		
			48.4	4.20		-	0	0
	146.4	86.36	52.9	3.30	-	-		

GG♂: Male giant grouper; TG♀: Female tiger grouper

day, 10 individual fresh fish that were used to feed the broodstocks were collected randomly, before the eyes and brains were pooled for detection of NNV.

Samples Collection from Produced Fry, Live Feed and Commercial Pellet

In NNV's vertical transmission study, three breeding attempts of different NNV status of broodstocks, which were 1) NNV-positive GG × NNV-positive TG (GG+ × TG+); 2) NNV-positive GG × NNV-negative TG (GG+ × TG-); and 3) NNV-negative GG × NNV-negative TG (GG- × TG-) were conducted as detailed above. Broodstocks of two GG and three TG with different status of NNV as determined earlier were used for these experiments. Following fertilization,

the obtained HG fry was reared and cultured in separated tanks until 120 days post-hatch (dph).

The management and rearing procedures for the newly hatched HG fry were conducted as previously described (Ch'ng & Senoo, 2008; Sufian & Nik-Haiha, 2015). After fertilization, each batch of the fry was placed into separate tanks. At 5, 10 and 20 dph, ~1000 whole body fry in each batch were sampled to detect the presence of NNV. The samples were divided into five different replicate tubes (200 fry/replicate). However, on days 30, 40, 60, 90 and 120 post-hatching, only the eyes and brains were collected from 150 juveniles. The samples were pooled and divided into five different replicates (50 juveniles/replicate). Twenty individuals at each sampling times were collected for total length and body weight measurements.

Moreover, on days 5, 10, 20 and 30 post-hatching, the rotifer that was used as live feed for the fry were also sampled at a rate of ~1000 rotifer/tube in five replicates for the detection of NNV. The commercial fish pellet that fed to the fry from 40 to 120 dph were also detected for NNV.

Water Quality Determination

Water temperature, pH, dissolved oxygen, salinity, conductivity and dissolved particles were measured *in situ* using an YSI 556 MPS probe (YSI Incorporation, NY, USA). The water sample was collected in sterilized polyethylene sampling bottles in replicates from two points within the hatchery, which were tanks containing the broodstocks and the newly produced HG fry. The levels of iron, ammonia, nitrate, nitrite, and phosphate were measured using a DR2800 spectrophotometer (Hach Company, Loveland, USA). The water qualities were determined every time prior to sampling of the broodstocks egg, sperm, and post-hatch HG fry.

Detection of NNV

In order to detect the presence of NNV, the total RNA of the eggs and sperm, fresh fish, live feed, and the HG fry were extracted using Viral RNA Mini Kit (Invitrogen, California, USA), according to the manufacturer's instructions. RT-PCR was performed using MyTaq™ One Step RT-PCR kit and MyTaq™ kit (Bioline, London, UK). The RT-PCR was carried out according to previously outlined methods (Nishizawa et al., 1994; World Organisation for Animal Health [OIE], 2017). The primers used were forward primer F2 (5' - CGTGTCAGTCATGTGTCGCT-3') and reverse primer R3 (5'-CGAGTCAACACGGGTGAAGA-3'). The PCR amplifications were performed using an Eppendorf Mastercycler Pro Thermal Cycler (Eppendorf, Hamburg, Germany) with reverse transcription at 45°C for 20 minutes, polymerase activation at 95°C for 1 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds and extension at 72°C for 30 seconds. After that, the first PCR product was later subjected for nested PCR using primer set of RGNNV-NFRG (5' - ACCTGAGGAGACTACCGCTC

- 3') and RGNNV-NRRG (5' - CAGCGAAACCAGCCTGCAGG - 3') as described by Nishioka et al. (2010). The amplification of cDNA was performed for one cycle at 95°C for 1 minute, 35 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 15 seconds, and extension at 72°C for 10 seconds. Then, the PCR product was electrophoresed with 1.5% agarose gel.

Sequencing and Data Analysis

A total of nine purified PCR products of NNV were used for sequencing (First Base, Kuala Lumpur, Malaysia). The nucleotide sequences of the NNV were compared with the known sequences in the GenBank database using Nucleotide Basic Local Alignment Search Tool (BLAST) program. Phylogenetic tree for NNV was generated by Neighborjoining of the MEGA 6.06 software (Tamura et al., 2013).

Pearson's correlation coefficient (*r*) (Statistix 9, Analytical Software, Tallahassee, FL, USA) was used to determine the possible correlation between the mean individual of water quality parameters in each sampling time with the presence of NNV. A p value at <0.05 indicates statistical significance.

RESULTS

Prevalence of NNV in Broodstocks

The prevalence of NNV among broodstocks between March 2016 and April 2017 is shown in Table 1. Low rate of NNV was detected among the broodstocks in the hatchery. The NNV was detected among broodstocks only in April 2016 in 50% of the males and 33.3% of the females. However, the NNV-positive broodstocks showed no symptom or clinical sign indicating of NNV infection. The nested PCR confirmed the detection with amplification of the 280 bp band (Figure 1).

Associations between Water Quality and NNV

The associations between the water quality with the detection of NNV among broodstocks and HG fry are presented

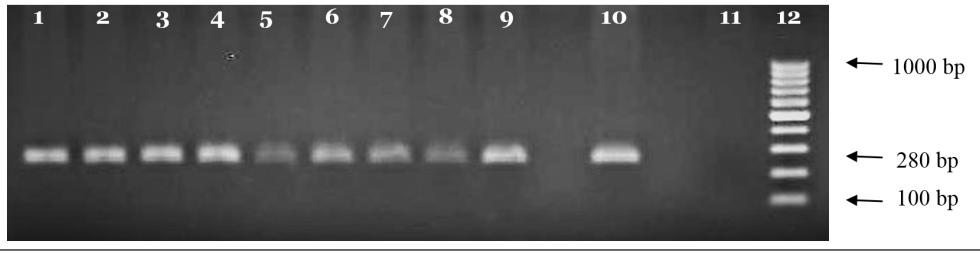

Figure 1. Detection of NNV in GG and TG broodstocks, and produced HG fry at different day post-hatch. Lane 1: GG ♂ broodstock; Lane 2: TG ♀ broodstock; Lane 3: Juvenile 40 dph (GG+ × TG-); Lane 4: Juvenile 40 dph (GG+ × TG+); Lane 5: Juvenile 60 dph (GG+ × TG-); Lane 6: Juvenile 90 dph (GG+ × TG-); Lane 7: Juvenile 90 dph (GG+ × TG+); Lane 8: Juvenile 120 dph (GG+ × TG-); Lane 9: Juvenile 120 dph (GG+ × TG+); Lane 10: Positive control of NNV (Abdullah et al., 2017); Lane 11: Negative control of NNV; Lane 12: 100 bp molecular weight marker (Fermentas). GG: Giant grouper; TG: Tiger grouper; +: positive to NNV; -: negative to NNV; ×: crossbreed; dph: days post-hatch

in Table 2 (Full water quality data in Appendices 1 to 5). Iron and ammonia consistently showed significant ($p<0.05$) and positive correlations with the detection of NNV in broodstocks of GG, TG, and GG+ × TG+ fry, while other parameters such as conductivity, dissolved oxygen, dissolved particles, nitrite, salinity and pH also showed significant positive/negative correlations with the detection of NNV, but without consistency.

Transmission of NNV to Produced HG's Fry

High rate of NNV detection between 60% and 100% were observed among HG fry produced from parents that at least one positive to NNV (Table 3). However, the NNV-positive HG fry showed no symptom or clinical sign indicating of NNV infection. No detection of NNV was also observed in fry that were produced by parent that both tested negative to NNV (Figure 1). Throughout the study period, NNV was not detected in the fresh fish, live feed, and commercial fish pellets.

Phylogenetic Tree Analysis

Analysis of the phylogenetic tree revealed that the detected NNV strains in this study were closely related to each other but distinctively grouped from other strains of NNV, including those isolated from east Malaysia (HQ859945 and HQ859922), other virus strains such as *Iridovirus* (DM015883.1), and the lymphocytes disease virus (KJ408273). The nucleotide sequences for NNV strains in this study, including from the broodstocks and the HG fry (40 dph to 120 dph) were deposited into the GenBank database with accession number from MG581289 to MG581297 (Figure 2).

Table 2
Relationships between the water qualities with the detection of NNV in grouper's broodstocks and produced HG fry from different NNV status of broodstocks

Water Quality	***r* value**				
	GG♂	**TG♀**	**GG+ × TG-**	**GG- × TG-**	**GG+ × TG+**
Conductivity (µS/cm)	0.7847	-0.9840*	0.8491	NA	-0.8646
Dissolve oxygen (mg/L)	0.7594	0.9548*	-0.9648	NA	-0.9795
Dissolve particles (mg/L)	0.8437*	-0.8854	0.6201	NA	0.4329
Iron (mg/L)	0.8783*	0.9649*	0.8660	NA	1.0000*
Ammonia (mg/L)	0.8731*	0.9573*	0.8885	NA	1.0000*
Nitrate (mg/L)	0.2740	0.9428	-0.0822	NA	-0.5000
Nitrite (mg/L)	0.8648*	0.9661*	0.0000	NA	0.8660
Salinity (ppt)	-0.9769*	-0.8193	0.8833	NA	0.9377
Temperature (°C)	-0.4995	-0.8430	0.7587	NA	0.3346
pH (1-14)	-0.9559*	-0.6572	0.9784	NA	0.0251

GG♂: Giant grouper; TG♀: Tiger grouper; +: positive to NNV; -: negative to NNV; ×: crossbreed; *: indicate statistically significant at $p < 0.05$

Table 3
Rate of detection of NNV in broodstocks and HG fry at different days post-hatch and the feed

Days Post-hatch	GG+ × TG+			GG+ × TG-			GG- × TG-			Fresh Fish	Live Feed (rotifer)	Pellet
	Length (cm)	Weight (g)	NNV Status (%)	Length (cm)	Weight (g)	NNV Status (%)	Length (cm)	Weight (g)	NNV Status (%)			
GG broodstock	140.0	70000	+	140.0	70000	+	150.0	80000	-	-	ND	ND
TG broodstock	46.0	2070	+	48.0	2130	-	73.0	5540	-	-	ND	ND
5	2.81 ± 0.51	0.15 ± 0.05	+ (100)	2.71 ± 1.08	0.11 ± 0.05	+ (100)	2.81 ± 0.32	0.18 ± 0.05	-	ND	-	ND
10	2.90 ± 1.23	0.39 ± 0.09	+ (100)	2.92 ± 1.19	0.35 ± 0.15	+ (60)	3.00 ± 0.55	0.47 ± 0.03	-	ND	-	ND
20	3.91 ± 1.09	1.63 ± 0.27	+ (80)	4.11 ± 1.34	1.57 ± 0.76	+ (100)	4.31 ± 0.53	1.69 ± 0.11	-	ND	-	ND
30	5.83 ± 1.33	3.74 ± 1.25	+ (100)	5.60 ± 1.31	3.69 ± 1.22	+ (80)	6.08 ± 0.67	4.07 ± 1.23	-	ND	-	ND
40	7.72 ± 1.04	16.23 ± 1.36	+ (80)	7.22 ± 1.57	11.54 ± 2.48	+ (80)	7.81 ± 0.89	17.02 ± 2.54	-	ND	ND	-
60	8.91 ± 1.22	61.11 ± 2.33	+ (80)	8.32 ± 1.88	56.41 ± 2.65	+ (80)	9.05 ± 0.12	69.85 ± 2.66	-	ND	ND	-
90	11.11 ± 1.34	132.11 ± 3.34	+ (100)	10.41 ± 1.69	120.00 ± 4.67	+ (100)	11.50 ± 0.40	151.43 ± 4.58	-	ND	ND	-
120	12.60 ± 1.25	250.00 ± 2.47	+ (80)	11.91 ± 2.64	220.00 ± 3.87	+ (100)	12.90 ± 0.54	289.00 ± 4.66	-	ND	ND	-

GG: Giant grouper; TG: Tiger grouper; +: positive to NNV; -: negative to NNV; ×: crossbreed; ND: not determined. The detection rate of NNV in fresh fish, live feed and commercial fish pellet were similar for all experiments

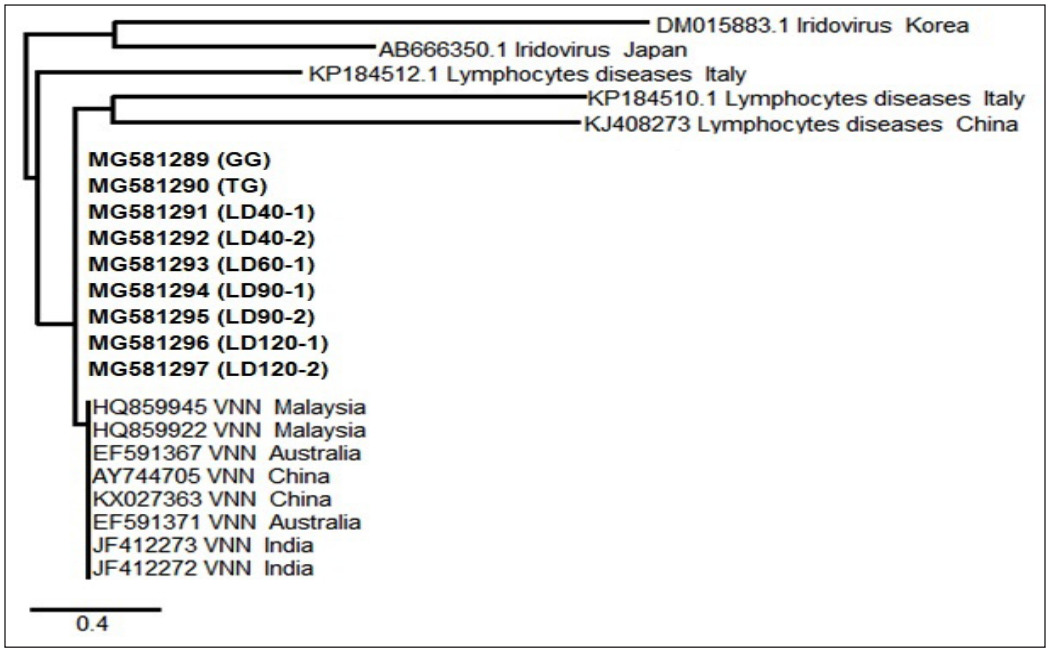

Figure 2. Phylogenetic tree showing the relationship between the NNV strains from this study with other NNV strains and fish viruses. NNV strains in this study were closely related between each other and distinctively grouped from other strains, including from east Malaysia (HQ859945 and HQ859922).GG: giant grouper; TG: tiger grouper; LD: Produced hybrid grouper fry whether at 40, 60, 90 and 120 days post-hatch

DISCUSSION

The prevalence of NNV among broodstocks in this hatchery was low. However, when at least one of the broodstocks was positive to NNV during breeding, all produced HG fry were NNV-positive at high prevalence. NNV is known as a disease that mainly affects the larvae and juveniles of cultured fish, resulting in high mortalities (Kokawa et al., 2008; Muroga 2001). However, under suitable conditions, sub-adults, market-size and adult fish (including broodstocks) can also be affected (Yanong 2016). Since this study was done under a non-stressful environment and no symptom or clinical sign of NNV infection was observed, the virus might shed from the broodstocks and the produced HG fry (Costa & Thompson, 2016). Moreover, the combination of RT and nested PCR methods used in this study were very sensitive and capable to identify the detected *Betanodavirus* as RGNNV genotype, which were usually isolated from warm-water fishes (Nishioka et al., 2010; OIE, 2017). In addition, without disease outbreak as observed in this study period, the high prevalence of NNV in HG fry is an important alert to the hatchery operators and farmers to consider.

This study revealed that iron and ammonia levels consistently showed significant correlations with the detection of NNV in broodstocks and GG+ × TG+ fry. Therefore, in order to reduce the risk of NNV infection, hatchery operators should closely monitor the two water quality

parameters, especially during the larval and fry periods. Moreover, water temperature has also been reported to be a risk factor in NNV infection in fish (Iwamoto et al., 2000; Yuasa et al., 2007). In this study, fresh fish, live, and commercial feed tested negative for NNV. Contrarily, previous studies in other countries showed that trash fishes are the main sources of betanodaviruses in cultured fish, and that they posed a serious risk for outbreaks of NNV in susceptible cultured fish (Doan et al., 2017; Gomez et al., 2010).

Phylogenetic tree analysis revealed possible vertical and horizontal transmissions of NNV in this hatchery. The virus might be transmitted horizontally among the broodstocks and fry. Similarly, vertical transmission occurred between broodstocks and fry, since infected broodstocks were most likely to transmit the virus to their respective fry. Furthermore, the NNV strains detected in this study were closely related with each other and distinctively grouped from other NNV strains, including from east Malaysia, suggesting the same virus strain is circulating within the hatchery system. Thus, the implementation of biosecurity measures is an important step towards controlling the disease.

CONCLUSION

The results of this study revealed the field scenario of the prevalence, risk factors, and transmission of NNV in a real hatchery environment. Hatchery operators are recommended to screen their broodstocks and produced fry before introduced into grow out farms in order to reduce the disease transmission and economic losses due to NNV. It is also expected that with the information obtained, a thorough biosecurity measure could be formulated and implemented to control NNV in the hatchery.

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APPENDIX

Appendix 1
*Water quality during the culture period of male GG (*Epinephelus lanceolatus*) broodstocks*

Month and Year	Temperature (°C)	pH (1-14)	Dissolved Oxygen (mg/l)	Salinity (ppt)	Conductivity (uS/cm)	Dissolved Particles (mg/l)	Ammonia (mg/l)	Nitrite (mg/l)	Nitrate (mg/l)	Phosphate (mg/l)	Iron (mg/l)
Mar 16	27.20±4.74	7.95±0.07	5.00±0.29	29.89±0.27	49.52±1.71	30.15±0.29	0.32±0.13	0.058±0.042	1.23±0.08	2.29±0.97	0.03±0.01
April 16	29.02±1.69	7.92±0.09	5.00±1.12	28.55±2.13	47.86±4.68	28.85±1.93	0.65±0.13	0.011±0.001	1.05±0.13	0.68±0.08	0.05±0.01
July 16	30.00±0.02	8.12±0.01	4.05±0.00	31.02±0.01	51.69±0.03	30.88±0.00	0.34±0.01	0.009±0.001	1.05±0.07	0.50±0.00	0.33±0.39
Aug 16	30.67±0.01	8.19±0.01	4.97±0.00	30.26±0.01	52.01±0.01	29.58±0.04	0.33±0.01	0.005±0.001	0.15±0.07	0.425±0.02	0.02±0.01
Sept 16	30.51±0.00	7.91±0.00	4.12±0.01	29.66±0.01	52.31±0.01	30.91±0.01	0.28±0.03	0.011±0.001	1.40±0.14	0.40±0.00	0.03±0.00
Oct 16	30.26±0.63	8.07±0.14	4.77±0.44	30.19±0.08	51.79±0.40	35.38±11.01	0.34±0.06	0.003±0.002	0.30±0.18	0.31±0.15	0.03±0.01
Nov 16	29.72±0.08	8.00±0.33	4.09±0.03	30.91±0.01	52.00±1.32	30.68±0.62	0.18±0.01	0.008±0.004	1.10±0.14	1.75±0.21	0.04±0.01
Dec 16	30.56±0.01	8.02±0.01	4.68±0.01	30.12±0.01	51.45±0.01	30.22±0.02	0.3±0.00	0.004±0.000	0.30±0.00	0.41±0.01	0.01±0.00
Jan 17	29.95±0.01	7.37±0.06	4.37±0.09	31.42±0.04	52.99±0.01	29.90±0.01	0.32±0.04	0.005±0.000	1.10±0.00	1.05±0.07	0.045±0.01
Feb 17	28.71±0.01	8.36±0.04	5.17±0.00	28.64±0.01	47.66±0.02	28.91±0.00	0.24±0.01	0.001±0.000	1.50±0.00	0.39±0.01	0.08±0.00
Mar 17	29.89±0.65	8.12±0.03	4.91±0.67	29.58±0.66	51.03±0.19	29.55±0.69	0.06±0.02	0.003±0.002	1.03±0.24	0.68±0.66	0.03±0.02
April 17	30.25±0.07	8.19±0.02	5.66±0.00	30.2±0.00	50.45±0.07	30.33±0.01	0.05±0.01	0.004±0.000	1.15±0.07	0.09±0.01	0.01±0.00

Appendix 2
*Water quality during the culture period of female TG (*Epinephelus fuscoguttatus*) broodstocks*

Month and Year	Temperature (°C)	pH (1-14)	Dissolved Oxygen (mg/l)	Salinity (ppt)	Conductivity (uS/cm)	Dissolved Particles (mg/l)	Ammonia (mg/l)	Nitrite (mg/l)	Nitrate (mg/l)	Phosphate (mg/l)	Iron (mg/l)
Mar 16	27.20±4.74	7.95±0.07	5.00±0.29	29.89±0.27	49.52±1.71	30.15±0.29	0.32±0.13	0.058±0.042	1.23±0.08	2.29±0.97	0.03±0.01
April 16	29.02±1.69	7.92±0.09	5.00±1.12	28.55±2.13	47.86±4.68	28.85±1.93	0.65±0.13	0.011±0.001	1.05±0.13	0.68±0.08	0.05±0.01
July 16	30.00±0.02	8.12±0.01	4.05±0.00	31.02±0.01	51.69±0.03	30.88±0.00	0.34±0.01	0.009±0.001	1.05±0.07	0.50±0.00	0.33±0.39
Aug 16	30.67±0.01	8.19±0.01	4.97±0.00	30.26±0.01	52.01±0.01	29.58±0.04	0.33±0.01	0.005±0.001	0.15±0.07	0.425±0.02	0.02±0.01
Sept 16	30.51±0.00	7.91±0.00	4.12±0.01	29.66±0.01	52.31±0.01	30.91±0.01	0.28±0.03	0.011±0.001	1.40±0.14	0.40±0.00	0.03±0.00
Oct 16	30.26±0.63	8.07±0.14	4.77±0.44	30.19±0.08	51.79±0.40	35.38±11.01	0.34±0.06	0.003±0.002	0.30±0.18	0.31±0.15	0.03±0.01
Nov 16	29.72±0.08	8.00±0.33	4.09±0.03	30.91±0.01	52.00±1.32	30.68±0.62	0.18±0.01	0.008±0.004	1.10±0.14	1.75±0.21	0.04±0.01
Dec 16	30.56±0.01	8.02±0.01	4.68±0.01	30.12±0.01	51.45±0.01	30.22±0.02	0.3±0.00	0.004±0.000	0.30±0.00	0.41±0.01	0.01±0.00
Jan 17	29.95±0.01	7.37±0.06	4.37±0.09	31.42±0.04	52.99±0.01	29.90±0.01	0.32±0.04	0.005±0.000	1.10±0.00	1.05±0.07	0.045±0.01
Feb 17	28.71±0.01	8.36±0.04	5.17±0.00	28.64±0.01	47.66±0.02	28.91±0.00	0.24±0.01	0.001±0.000	1.50±0.00	0.39±0.01	0.08±0.00
Mar 17	29.89±0.65	8.12±0.03	4.91±0.67	29.58±0.66	51.03±0.19	29.55±0.69	0.06±0.02	0.003±0.002	1.03±0.24	0.68±0.66	0.03±0.02
April 17	30.25±0.07	8.19±0.02	5.66±0.00	30.2±0.00	50.45±0.07	30.33±0.01	0.05±0.01	0.004±0.000	1.15±0.07	0.09±0.01	0.01±0.00

Appendix 3

Water quality during the culture period of GG+ × TG- fry

Days Post Hatch	Temperature (°C)	pH (1-14)	Dissolved Oxygen (mg/l)	Salinity (ppt)	Conductivity (uS/cm)	Dissolved Particles (mg/l)	Ammonia (mg/l)	Nitrite (mg/l)	Nitrate (mg/l)	Phosphate (mg/l)	Iron (mg/l)
5	27.48±0.00	7.90±0.01	5.24±0.03	26.81±0.01	43.86±0.01	27.22±0.01	0.02±0.00	0.012±0.001	0.90±0.00	0.50±0.01	0.06±0.00
10	27.43±0.00	7.85±0.00	4.71±0.01	26.76±0.01	43.76±0.02	27.19±0.01	0.02±0.00	0.008±0.001	0.95±0.21	0.26±0.00	0.05±0.01
20	28.71±0.01	8.36±0.04	5.17±0.00	28.64±0.01	47.66±0.02	28.91±0.00	0.01±0.00	0.001±0.000	1.50±0.00	0.22±0.01	0.08±0.00
30	28.43±0.00	8.04±0.01	5.13±0.03	28.65±0.01	47.39±0.00	28.90±0.02	0.01±0.00	0.003±0.001	0.95±0.21	0.14±0.01	0.01±0.01
40	30.19±0.49	8.26±0.01	4.78±0.02	31.04±0.05	53.05±0.01	31.04±0.08	0.09±0.00	0.005±0.001	1.50±0.14	0.25±0.01	0.05±0.00
60	30.20±0.01	7.99±0.01	4.79±0.12	29.71±0.01	50.63±0.04	29.93±0.01	0.18±0.01	0.007±0.001	1.30±0.00	0.54±0.01	0.03±0.01
90	31.10±0.23	8.22±0.01	3.88±0.04	30.91±0.01	53.52±0.00	31.02±0.11	0.38±0.09	0.005±0.001	1.10±0.14	0.21±0.00	0.08±0.01
120	28.74±0.00	8.25±0.00	4.35±0.00	29.01±0.01	48.23±0.01	30.12±0.01	0.38±0.01	0.005±0.000	1.55±0.07	0.20±0.01	0.05±0.00

Appendix 4

Water quality during the culture period of GG+ × TG+ fry

Days Post Hatch	Temperature (°C)	pH (1-14)	Dissolved Oxygen (mg/l)	Salinity (ppt)	Conductivity (uS/cm)	Dissolved particles (mg/l)	Ammonia (mg/l)	Nitrite (mg/l)	Nitrate (mg/l)	Phosphate (mg/l)	Iron (mg/l)
5	27.55±0.00	7.84±0.01	5.97±0.11	26.70±0.06	43.81±0.09	27.18±0.04	0.05±0.01	0.010±0.001	1.00±0.14	0.46±0.01	0.05±0.01
10	27.57±0.01	7.86±0.01	3.72±0.05	26.78±0.00	43.90±0.00	27.24±0.00	0.04±0.00	0.005±0.000	0.95±0.07	0.42±0.01	0.04±0.01
20	28.70±0.01	8.23±0.00	4.66±0.01	28.68±0.00	47.59±0.02	28.91±0.02	0.06±0.01	0.002±0.001	1.10±0.00	0.15±0.03	0.02±0.01
30	28.74±0.01	8.24±0.01	4.37±0.01	28.98±0.01	48.24±0.01	29.24±0.00	0.02±0.00	0.006±0.001	1.65±0.21	0.20±0.01	0.05±0.00
40	30.31±0.00	8.00±0.02	4.83±0.11	29.72±0.00	50.65±0.00	29.98±0.05	0.06±0.01	0.005±0.001	1.25±0.07	0.65±0.01	0.02±0.00
60	30.01±0.01	7.74±0.01	4.81±0.01	30.03±0.04	50.57±0.71	30.24±0.01	0.26±0.04	0.011±0.001	1.25±0.07	0.15±0.01	0.05±0.02
90	30.48±0.00	8.00±0.02	4.02±0.01	30.40±0.02	51.92±0.02	30.52±0.03	0.55±0.02	0.005±0.000	1.10±0.14	0.13±0.01	0.05±0.00
120	30.00±0.00	7.34±0.01	4.03±0.01	31.63±0.05	44.56±0.00	29.89±0.01	0.24±0.01	0.005±0.001	0.95±0.07	0.09±0.00	0.04±0.01

Appendix 5

Water quality during the culture period of GG- × TG- fry

Days Post Hatch	Temperature (°C)	pH (1-14)	Dissolved Oxygen (mg/l)	Salinity (ppt)	Conductivity (uS/cm)	Dissolved Particles (mg/l)	Ammonia (mg/l)	Nitrite (mg/l)	Nitrate (mg/l)	Phosphate (mg/l)	Iron (mg/l)
5	28.71±0.01	8.36±0.04	5.17±0.00	28.64±0.01	47.66±0.02	28.91±0.00	0.03±0.01	0.001±0.000	1.50±0.00	0.39±0.01	0.08±0.00
10	29.95±0.01	7.37±0.06	4.37±0.09	31.42±0.04	52.98±0.01	29.90±0.01	0.01±0.00	0.005±0.000	1.10±0.00	0.25±0.02	0.05±0.01
20	29.36±0.00	7.24±0.01	5.24±0.04	30.85±0.00	52.94±0.01	28.92±0.00	0.05±0.00	0.003±0.000	1.15±0.07	0.11±0.01	0.01±0.00
30	31.06±0.01	7.89±0.01	4.89±0.02	30.77±0.01	51.15±0.00	28.90±0.02	0.02±0.00	0.001±0.000	1.05±0.07	0.31±0.01	0.03±0.01
40	30.25±0.01	7.58±0.01	5.26±0.00	30.11±0.01	50.57±0.07	29.72±0.00	0.04±0.00	0.005±0.001	1.45±0.07	0.35±0.01	0.03±0.02
60	30.21±0.01	7.43±0.01	4.79±0.01	30.27±0.01	46.66±0.00	26.70±0.06	0.02±0.01	0.002±0.001	1.20±0.00	0.17±0.01	0.01±0.00
90	31.45±0.01	7.25±0.00	4.58±0.02	30.85±0.01	50.10±0.00	29.24±0.00	0.02±0.00	0.004±0.000	1.00±0.00	0.32±0.01	0.04±0.01
120	31.13±0.03	7.44±0.00	4.93±0.02	31.02±0.00	48.45±0.01	30.02±0.00	0.01±0.00	0.001±0.000	1.30±0.14	0.19±0.00	0.01±0.00

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α-glucosidase Inhibitory and Antioxidant Activities of *Entada spiralis* Ridl. (Sintok) Stem Bark Extracts

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ABSTRACT

Entada spiralis Ridl. (Leguminosae), locally known as Sintok or Beluru, is a tropical woody climber that grows widely in Malaysia. It is a valuable and well-known plant in herbal medicine due to its various traditional and medicinal applications. Crude extracts were obtained from the stem bark by using petroleum ether, chloroform, and methanol as extracting solvents and were then bioassayed for their biological potential. The antioxidant and α-glucosidase inhibitory activities of the extracts were assessed by using DPPH, ABTS, β-carotene, and α-glucosidase inhibitory methods. Qualitative analysis showed the presence of most of the phytochemicals in methanol extract; however, chloroform and petroleum ether extracts contained terpenoid and tannins as their major phytoconstituents, respectively. The methanol extract contained the highest amount of total phenolics (42.5 ± 15.85 µg GAE/mg) and flavonoids (28.94 ± 2.93 µg QE/mg), and showed the most potent α-glucosidase inhibitory activity with an IC_{50} value of 20.67 µg/mL. The same methanol extract exhibited the highest β-carotene bleaching inhibition (27% at 1 mg/mL), while methanol and chloroform extracts exhibited good radical scavenging activities (IC_{50} 37.29 ± 0.05 and 90.84 ± 3.12 µg/mL, respectively) against ABTS and DPPH radicals. Bioassay-guided silica gel column chromatography purification of the most active methanol extract afforded 3, 4′,5,7-tetrahydroxyflavone (6 mg). The compound displayed promising inhibitory activities against free radicals as well as α-glucosidase enzyme. These results suggest the potential use of *E. spiralis* Ridl. stem bark as a therapeutic agent against hyperglycaemia.

Keywords: Antioxidant, α-glucosidase, β-carotene, crude extracts, DPPH, *Entada spiralis* Ridl., Leguminosae, 3,4′,5,7-tetrahydroxyflavone

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INTRODUCTION

Plants continue to play a vital role in the healthcare system. More than two thirds of the world population depend on medicinal plants for their primary healthcare (World Health Organization [WHO], 2009) and this has served as a catalyst for continuous research on traditional plants which has resulted in the discovery of several plant-derived drugs and active compounds that are used directly in medicine. It is assumed that almost 75 percent of the world's existing plant species have medicinal value and nearly all of these plants possess potent antioxidant potential (Krishnaiah et al., 2011). This potential is the result of the presence of both low and high molecular weight secondary metabolites which are derived or synthesised from primary metabolites such as sugars and amino acids through glycosylation, hydroxylation, and methylation (Kasote et al., 2015).

α-glucosidase is a membrane-bound enzyme situated at the gut wall of the small intestine. It catalyses the hydrolysis of terminal α-(1-4)-linked glucose, releasing a single α-glucose molecule (Chiba, 1997). One of the most acceptable ways of treating diabetes is by reducing postprandial hyperglycaemia (Sudha et al., 2011; Xie et al., 2003). The only way to achieve this is by retarding the actions of digestive enzymes to delay the digestion and absorption of glucose through the brush border (Ahmed et al., 2017; Hilmi et al., 2014; Kazeem et al., 2013). Commercial enzyme inhibitors such as voglibose, acarbose, and miglitol, have been reported to be accompanied with serious gastrointestinal side effects like diarrhoea, flatulence and bloating (Deacon, 2011; Martin & Montgomery, 1996), and this has further increased the search for digestive enzyme inhibitors from natural sources. Several plant extracts have been reported to be powerful starch-hydrolysing enzyme inhibitors. These plant-derived inhibitors are more acceptable due to their low cost and less side effects (Benalla et al., 2010; Bhat et al., 2011).

Some chemical compounds and reactions generate free radicals or oxygen species (pro-oxidants), while some compounds and reactions, on the other hand, scavenge and oppose their toxic actions (antioxidants). In a normal cell, there is an optimal balance between pro-oxidants and antioxidants. However, when there is an increase in the generation of free radical or oxygen species against the level of antioxidant in the body, the balance shifts towards pro-oxidants and this results in oxidative stress. Free radicals have been considered to be the major causative agents of cell damage, causing diseases such as diabetes mellitus, cancer, cardiovascular and liver problems (Boligon et al., 2014; Hasan et al., 2017; Yankuzo et al., 2011). Antioxidants are therefore needed to stabilise and neutralise free radicals to prevent them from attacking cells and tissues. There has been a global interest in plant-derived antioxidants because of their high efficacy and relatively less side effects (Dehghan, et al., 2016; Sarian et al., 2017).

Entada (synonym: *Entadopsis* Britton) belongs to the pea family of Leguminosae containing approximately 28 species,

with about six species found in Asia, 21 species in Africa, and two in America. Various enzyme-inhibiting and antioxidant activities of different species of *Entada* have been reported in the literature. Several compounds isolated from seed kernel of *Entada scandens*, whole plantlets of *Entada africana* and *Entada abyssinica* contain high antioxidant activity (Guissou et al., 2010: Teke et al., 2011). Ethanol extract of *Entada rheedii* seed coat, cotyledon, and pericarp demonstrated significant α-glucosidase inhibition with IC_{50} values of 98.73 ± 0.46, 28.08 ± 11.28, and 74.01 ± 2.02 mg/ mL, respectively (Ruangrungsi et al., 2008). Acidified methanol and 70% acetone extracts of *E. scandens* seed also displayed good α-amylase inhibitory potential of 89% and 34.82%, respectively (Gautam et al., 2012).

In Malaysia, the most common species of *Entada* is *Entada spiralis* Ridl (synonym: *Entada scheffleri*), locally known as Beluru or Sintok. It is a woody climber that can grow up to a height of 25 m. Sintok possesses a wide range of ethnomedicinal uses. The root decoction is used to treat venereal diseases and haemorrhoids while the stem bark is used for hair treatment, for cleaning insect bites and also used as body wash (Harun et al., 2015). A previous study on this plant showed the potency of the methanol fraction against human dermatophyte (Harun et al., 2011). However, despite the fact that many species from these genera have been reported by previous researchers as having a positive effect on body glucose level and enhancing antioxidant capacity, so far, no research has been conducted on *E. spiralis*, particularly on its potential as a digestive enzyme inhibitor and as an antioxidant agent. Hence, this study aimed to determine the α-glucosidase and antioxidant activities of different extracts of *E. spiralis* Ridl. stem bark.

MATERIALS AND METHODS

Chemicals and Reagents

Quercetin (QC), sodium carbonate (Na_2CO_3), aluminium chloride ($AlCl_3$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium persulfate, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), *p*-nitrophenyl glucopyranoside (*p*-NPG) and α-glucosidase enzyme from *Saccharomyces cerevisiae*, Tween 40, β-carotene, and linoleic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin–Ciocalteu reagent (FC), gallic acid, and ascorbic acid were obtained from Merck (Darmstadt, Germany). All chemicals and solvents used were of analytical grade.

General

FTIR spectrometer (Perkin Elmer Inc., Massachusetts, USA) equipped with horizontal attenuated total reflectance device was used to detect the functional groups. Melting point was measured using Smp 10, BIBBY STERILIN, Ltd, Stone, ST50SA, United Kingdom. UV-VIS spectrophotometer 1800 series, Shimadzu, Japan was used to detect the presence or

absence of chromophores. ^{1}H-, ^{13}C- NMR spectra were measured using FT-NMR cryoprobe Bruker advance 111 spectrometer (500 and 150 MHz, respectively), Bruker Scientific Technology Co., Ltd. Yokohama, Japan. Absorbance was measured using microplate Reader TECAN PRO 200, Tecan Trading AG, Switzerland. Chromatotron model 7924T (T-squared Technologhy, Inc), USA was used to purify the compound.

Plant Preparation and Extraction

Entada spiralis Ridl. stem barks were obtained from Tasik Chini Forest, Pekan District, Pahang, Malaysia (voucher specimen KMS-5228) were cut into smaller pieces, air-dried at room temperature, and pulverized into powdered form to give a final mass of 4.5 kg. The powder was macerated successively using petroleum ether, chloroform, and methanol to get petroleum ether extract (Ep), chloroform extract (Ec), and methanol extract (Em), respectively. Maceration with each solvent was repeated until exhaustion before proceeding to the next solvent and the resultant filtrates from each solvent were concentrated *in vacuo* using a rotary evaporator (IKA RV 10B S99, 40°C, 115 rpm) (Ahmed et al., 2012). The crude extracts were packed in a glass bottle and kept in the fridge until further analysis.

Fractionation and Purification of Methanol Extract: Isolation of Active Principle

Methanol extract (most active, 10 g) was subjected to silica gel (70–230 mesh) column chromatography (30 × 80 cm) and eluted with gradient solvent system of $CHCl_3$:MeOH 90%:10%–10%:90% to generate pooled fractions F1–F4. Dried F1 (1.7 g) was dissolved in ethanol to remove ethanol-insoluble portion. The ethanol-soluble portion (900 mg) was subjected to repeated centrifugal chromatography using silica gel PF254 with gypsum with the solvent system 90% DCM:10% $CHCl_3$ to afford C1 (6 mg) as the active principle.

Estimation of Phytoconstituents

Determination of Total Phenolic Content (TPC). The TPC in different *E. spiralis* extracts was determined using Folin–Ciocalteu method adapted from Ahmed et al. (2015) and Umar et al. (2010) with some modifications. Gallic acid was used as standard. 50 µL of 10% FC w/v (FC: H_2O) was introduced into a 96-well microplate followed by 10 µL of standard or sample (7.18–1000 µg/mL). Blanks contained only the solvent. 50 µL of 40% Na_2CO_3 was then added to each well and the plate was incubated for 2 h at room temperature. Absorbance was measured at 725 nm using a microplate reader. The assay was conducted in triplicate and total phenolic content was determined from the linear regression curve of absorbance against concentration. Results obtained were expressed as microgram of gallic acid equivalence per milligram dry weight of the extract (µg GAE/mg dw of extract).

Determination of Total Flavonoid Content (TFC). The methods reported by Abdel-Hameed (2009) and Ahmed et al. (2015)

were followed with slight modifications. Quercetin (QC) was used as standard. 100 µL of 2% $AlCl_3$ in methanol was added to 100 µL of extract (1 mg/mL) or standard (7.81–250 µg/mL). Blanks contained extracts with solvent without $AlCl_3$. Absorbance was measured at 415 nm after 15 min. The test was conducted in triplicate and a quercetin calibration curve was used to determine the concentration of each extract using the equation Y = mx + c. Results were expressed as microgram of QU equivalence per milligram dry weight of the sample.

Antioxidant Activity

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Assay. This assay was conducted using DPPH by modifying methods from Ahmed et al. (2015) and Sulaimon et al. (2011). 150 µL of freshly prepared 0.4 M DPPH solution was carefully pipetted into a round-bottomed 96-well microplate. 100 µL of sample or standard with varying concentrations (7.81–500 µg/mL) were added. Blanks contained only the extraction solvent and DPPH. Ascorbic acid dissolved in distilled water and quercetin dissolved in MeOH were used as standards. The plate was left in the dark for 25 min to activate, after which it was placed in a microplate reader. Absorbance was read at 517 nm. The test was conducted in triplicate and the percentage inhibition of each sample/ standard was calculated using the following equation:

$$(\%\ DPPH\ inhibition) = \left[\left[\frac{Ac - As}{Ac}\right]\right] \times 100$$

where *Ac* represents the absorbance of DPPH radical in MeOH and *As* represents the absorbance of DPPH radical in the sample or standard. IC_{50} obtained from graphical plot from percentage inhibition against concentration was used to define the radial scavenging activity of each extract.

2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), ABTS Assay. For ABTS assay, the Zheleva-Dimitrova et al. (2010) method was adopted with some modifications. Stock solutions of ABTS (7 mM) and potassium persulfate (2.45 mM) were prepared using distilled water. The working solution was prepared by adding 1 mL of ABTS solution to an equal volume of potassium persulfate solution. The reaction mixture was left overnight for 16 h to generate the intense blue-coloured ABTS radical $ABTS^{\bullet+}$. $ABTS^{\bullet+}$ (1 mL) was added to 50 mL of MeOH and distributed (100 µL each) into a 96-well microplate containing 100 µL of serially diluted sample or standard (7.81-125 µg/mL). Absorbance was measured at 734 nm against blank (containing sample and MeOH only). The test was conducted in triplicate. The extracts were compared with Trolox and ascorbic acid as standards and percentage inhibition was calculated as follows:

$$(\%\ ABTS\ inhibition) = \left[\left[\frac{Ac - As}{Ac}\right]\right] \times 100$$

where *Ac* is the absorbance of ABTS in MeOH and *As* is the absorbance of ABTS in the sample or standard. The radical scavenging activity was determined from the

IC_{50} obtained from the percentage inhibition curve against different concentrations of the sample or standard.

β -Carotene Bleaching Assay. This assay was conducted according to Duan et al. (2006) and Yim et al. (2010) with slight modifications. Briefly, emulsion of β-carotene (BC) and linoleic acid (LA) was obtained by dissolving 200 µg BC in 1 mL chloroform. This was rapidly transferred into a 250 mL round bottomed flask containing 20 µL of LA and 200 µL of Tween 40. Chloroform was removed at 45°C using a rotary evaporator, after which 50 mL of hot distilled deionized water (50°C) mixed with 5 mL of phosphate buffer (pH 6.8) was added. The mixture was stirred vigorously to form BC–LA emulsion. A control emulsion was prepared without BC. Next, 200 µL of the emulsion was added into a round-bottomed 96-well microplate containing 50 µL of extract or standard (1 mg/mL). Initial absorbance at time 0 was measured immediately at 450 nm. Subsequent absorbance at time t was recorded at every 10 min for 1 h. Control was measured using solvent and control emulsion. Quercetin was used as standard. The percentage of BC bleaching inhibition was calculated using the following equation:

$$(\%\ bleaching\ inhibition) = \left[\left[\frac{Rc - Rs}{Rc}\right]\right] \times 100$$

where *Rc* and *Rs* are the bleaching rates for the control and sample, respectively.

α-Glucosidase Inhibitory Assay. Effect of extracts or compounds on the inhibition of α-glucosidase was determined according to the method described by Jeong et al. (2013) with minor modifications. Briefly, α-glucosidase enzyme obtained from *Saccharomyces cerevisiae* (1 U/mL) was dissolved in freshly prepared 50 mM K_3PO_4 buffer (pH 6.9) as a stock solution. The substrate, 5 mM *p*-nitrophenyl glucopyranoside (*p*-NPG), was prepared in 0.1 M phosphate buffer (pH 6). 100 µL of the enzyme was then transferred into a 96-well microplate containing 50 µL of sample or standard and incubated for 10 min at room temperature. Next, 50 µL of *p*-NPG was added and the plate was incubated for another 5 min. Absorbance was immediately measured at 405 nm using a microplate reader. Quercetin was used as standard. The percentage inhibition of α-glucosidase was calculated using the following equation:

$$(\%) = \left[\left[1 - \frac{S - b}{C}\right]\right] \times 100$$

where *S* is the absorbance of the sample or standard, *b* is the absorbance of the blank containing 100 µL of MeOH + 50 µL of sample + 50 µL of substrate, and *C* is the control containing 100 µL of buffer and 100 µL of enzyme. The percentage of the extract required to inhibit 50% of the α-glucosidase activity (IC_{50}) was determined from the regression curve. The experiment was conducted in triplicate.

Statistical Analysis

Data were analysed using one-way analysis of variance (ANOVA). Results were expressed as mean ± standard error of mean (SEM) of triplicate measurements. Significant differences between parameters were determined using Tukey's HSD post hoc tests (significant at $p<0.05$).

RESULTS AND DISCUSSION

Structural Characterization of Active Principle

Active principle (C1) was obtained as a yellow amorphous powder (m.p.: 275–277°C). It displayed UV absorption (λ_{max}) at 225 nm, indicating the presence of aromatic rings. IR spectrum showed absorptions at 3291 cm^{-1} for O–H stretch, 2924 cm^{-1} for C–H stretch, 1257 cm^{-1} for C–O stretching vibration. The vibration at 1613 cm^{-1} indicated olefinic C=C stretch. ^{13}C-NMR spectrum showed a total of thirteen carbon signals, suggesting a flavonoid skeleton comprising six aromatic CH and nine quaternary carbons with C=O appearing at δ_C 176.64 ppm. Complete ^{13}C-NMR and ^{1}H-NMR (500 MHz, MD_3OD) spectra data are given in Table 1. ^{1}H-NMR spectrum of C1 displayed signals comprising of two meta-coupled doublets at 6.21 and 6.42 ppm each with J = 2.1 and 2.2 Hz assignable to H-6 and H-8 of ring A of flavone skeleton, respectively. Signals for the B-ring protons appeared at 6.94 (dd, J= 2.0, 7.0, 2H, H-3′& H-5′) and 8.12 (dd, J= 2.0, 7.0, 2H, H-2′ & H-6′). ^{13}C-NMR and ^{1}H-NMR of C1 are shown in Figure 1 Spectra data obtained were compared with the literature (Ahmed et al., 2014) and consequently the isolated flavonoid (C1) was identified as 3,4′,5,7-tetrahydroxyflavone (Figure 1), a known flavonol (kaempferol), which has already been reported for its antioxidant and antidiabetic activities (Sarian et al., 2017).

Table 1
Spectra data of C1 compared with literature

Position	δ_H (ppm), m J (Hz)	δ_C (ppm)	δ_H (ppm), m J (Hz)	Kaempferol δ_C (ppm) (Ahmed et al., 2014)
2	-	146.70	-	147.12
3	-	135.72	-	136.75
4	-	176.64	-	176.71
5	-	161.09	-	162.44
6	6.21 (1H, d), J=2.1	97.94	6.27 (d), J = 1.8	99.26
7	-	164.31	-	165.05
8	6.42 (1H, d), J= 2.2	93.12	6.54 (d), J = 1.8	94.61
9	-	156.88	-	157.89
10	-	103.13	-	104.27
1'	-	122.35	-	123.44
2' & 6'	8.12 (2H, dd) J = 2.0, 7.0	129.29	8.16 (dd) J = 1.8, 8.7	130.58
3' & 5'	6.94 (2H, dd) J = 2.0, 7.0	114.93	6.54 (dd) J = 2.4, 9.0	116.26
4'	-	159.15	-	160.26

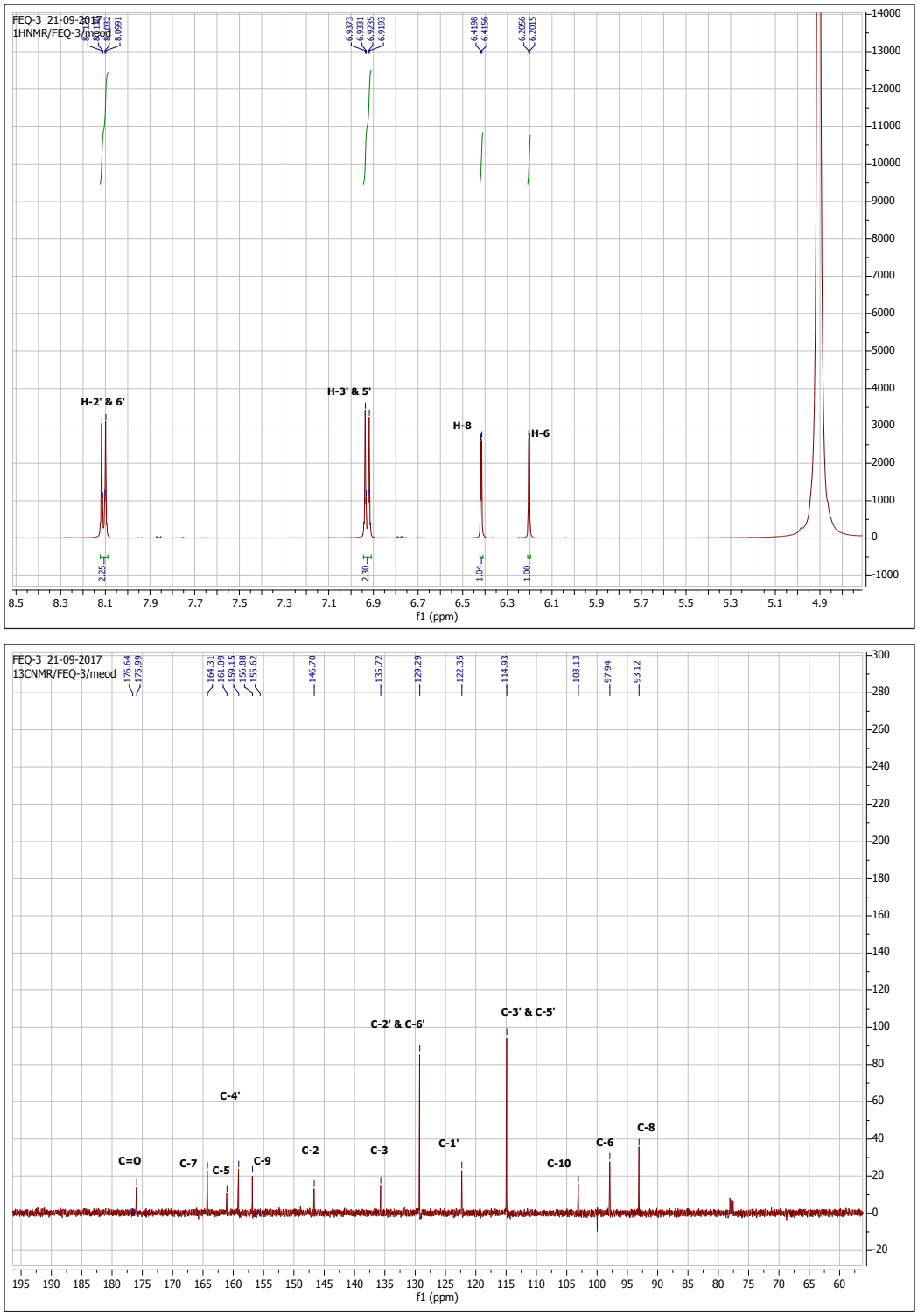

Figure 1. [1]H-NMR and [13]C-NMR spectra of C1

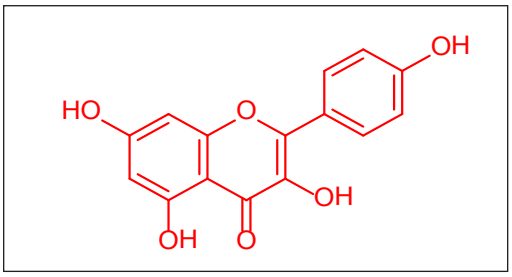

Figure 2. Structure of C1 isolated from active methanol extract of *E. spiralis*

Estimation of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Flavonoids are major phenolic compounds and are naturally occurring antioxidants. The biological activities, especially the free radical scavenging property of phenols, can be attributed to their reducing properties as a hydrogen or electron donor (Rice-Evans et al., 1997). Results obtained from TPC and TFC of different samples are shown in Table 2. TPC values were observed to vary significantly among the samples ($p < 0.05$). Methanol extract (Em) showed the highest value (42.5 ± 8.59 µg GAE/mg dw) as obtained from the calibration curve of gallic acid (R^2= 0.9941). Similar methanol extract from *E. pursaetha* seeds have been reported to possess 5.5 mg per catechol equivalent /g of the sample (Pakutharivu & Suriyavadhana, 2010). TPC of all *E. spiralis* extracts were observed in decreasing order from Em > Es > Ep with the values of 42.5 ± 8.58, 28.3 ± 1.38 and 2.6 ± 0.95 µg GAE/mg dw, respectively. Similarly, TFC followed the same trend. Overall, TFC values were observed to be lower than the TPC values. This is expected because flavonoids are a part of phenolic compounds.

Table 2
Total phenolic and flavonoid contents of various extracts of E. spiralis

Extract	TPC (µg GAE/mg dw)	TFC (µg QE/mg dw)
Em	42.56 ± 8.59	28.94 ± 2.93
Ec	28.30 ± 1.38	12.73 ± 1.93
Ep	2.62 ± 0.95	0.84 ± 0.24
R^2	0.9941	0.9747

Values are expressed as mean ± SEM (n=3) of triplicate measurements. Results were analysed using one-way ANOVA. Significant difference was determined using Tukey's HSD (at $p < 0.05$). TPC (total phenolic content), TFC (total flavonoid content), GAE (gallic acid equivalence), QE (quercetin equivalence), Em (*E. spiralis* methanol extract), Ec (*E. spiralis* chloroform extract), Ep (*E. spiralis* petroleum ether extract), dw (dry weight), R^2 (R-squared value obtained in each regression line with different concentrations of standards)

Antioxidant Activity

Radical Scavenging Activities of DPPH and ABTS. The maximum absorption of DPPH radical is 517 nm. This absorption diminishes when free radical is reduced to hydrazine derivatives by the action of antioxidants through the electron transfer or H-atom transfer process (Kosar et al., 2011). Also, the extent of decolourisation of $ABTS^{\bullet+}$ at 734 nm is used in measuring the antioxidant potential of extracts or individual compounds under investigation. Analysis of antioxidant activities of various extracts at different concentrations (7.81–125 µg/mL) on DPPH and ABTS radicals with

their corresponding IC_{50} values are shown in Table 3 with ascorbic acid (AC), Trolox (Tx) (ABTS only), and quercetin (QC) as reference standards. All the tested samples and standards showed radical scavenging activity in a concentration-dependent manner. For the DPPH method, inhibitory activities were observed in decreasing order from AC > QC > C1> Em > Ec > Ep.

Table 3
Radical scavenging activities of different extracts and compounds from E. spiralis *stem bark*

Sample	DPPH radical-scavenging IC_{50} (µg/mL)	ABTS radical-scavenging IC_{50} (µg/mL)
Em	42.67 ± 4.10^{C}	37.29 ± 0.05^{C}
Ec	472.83 ± 11.20^{D}	90.84 ± 3.12^{D}
Ep	1050.57 ± 23.21^{E}	232.08 ± 26.53^{E}
C1	31.69 ± 1.57^{A}	22.56 ± 3.15^{B}
QC	29.82 ± 3.73^{A}	-
Tx	-	15.23 ± 2.15^{A}
AC	24.67 ± 0.45^{B}	16.74 ± 1.76^{A}

Values are expressed as mean ± SEM of triplicate measurement. Results were analysed using one-way ANOVA. Significant difference was determined using Tukey's HSD (at $p < 0.05$). Em (*E. spiralis* methanol extract), Ec (*E. spiralis* chloroform extract), Ep (*E. spiralis* petroleum ether extract), Tx (Trolox), QC (quercetin), C1 (3,4′,5,7-tetrahydroxyflavone), AC (ascorbic acid), IC_{50} (concentration of a sample required to scavenge 50% of the free radicals). Values with the same letters are not significantly different.

The radical scavenging activity of C1 was statistically similar with QC (significance at $< p < 0.05$). All the extracts and the C1 showed better radical scavenging activity towards ABTS radicals compared to that of DPPH. The scavenging activity of Em for all concentrations (except at the lowest concentration) was significantly higher than Trolox ($p < 0.05$). There was no significant difference in the scavenging activity of Tx and AC (at $p < 0.05$). AC showed the highest and lowest radical scavenging activity against ABTS radical and hence, displayed the lowest IC_{50} (16.74 ± 1.76 µg/mL) while Ep showed the lowest activity and therefore, showed the highest IC_{50} value (232.08 ± 26.53 µg/mL). The lower activity exerted by Ec compared to Em could be attributed to the lesser amount of TPC and TFC in the extract since these contents are the major constituents responsible for antioxidant activity (Rice-Evans et al*.,* 1997). Similar lower activity of chloroform extract was reported from the root, leaves and stem bark of *E. africana*. All the chloroform extracts displayed higher EC_{50} values against DPPH radicals than that of methanol extracts (Tibiri et al*.,* 2010). The relatively low activities observed in scavenging potentials of Ep may be attributed to low content of phenolics in the extract (Oyedemi et al., 2013).

β-Carotene Bleaching Assay. This assay is based on the discolouration of β-carotene (BC) by a lipid or peroxy radical as a result of breakage of the π-conjugation by linoleic acid (LA) to C=C of BC, forming hydroperoxide free radical by auto-oxidation during incubation (at 50°C). However, the presence of an antioxidant retards this

reaction Duan et al., (2006). The extent of BC bleaching inhibitory activity of different extracts of *E. spiralis* and standard is shown in Figure 3. There was no significant difference in the inhibitory activity of Em and Ec, while Ep was observed to have the lowest activity. Meanwhile BC bleaching inhibition of *Polysiphonia urceolata* was reported to show inhibitory activity stronger than that of gallic acid used as positive control after 420 min (Duan et al*.,* 2006). Using the same reaction time, similar strong activity was observed in different edible mushrooms species (Barros et al*.,* 2007). This means that the relatively low activity displayed by all the extracts and control could be due to shortage in reaction time, suggesting that the time needs to be extended further to enhance maximum activity.

Values with different letters are significantly different ($p < 0.05$). Values are expressed as mean ± SEM of three determinations.

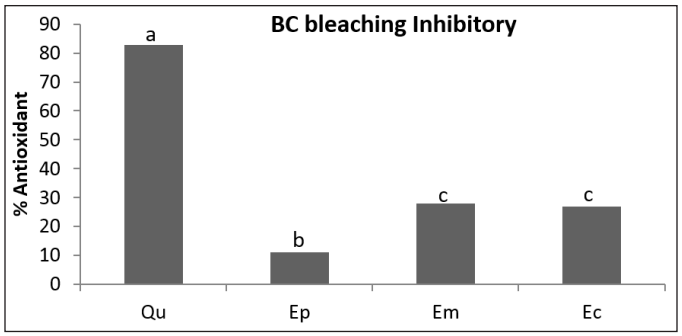

Figure 3. β-Carotene bleaching inhibitory activity of quercetin (Qu) and *E. spiralis* extracts

α-Glucosidase Inhibitory Assay. α-Glucosidase is a carbohydrate-hydrolysing enzyme responsible for postprandial hyperglycaemia (Kwon et al*.*, 2007). Its main function is to catalyse the hydrolysis of disaccharides to monosaccharides, which leads to a drastic increase in the level of glucose in the body (Matsui et al*.*, 2007).

Table 4
α-Glucosidase inhibitory activity of different extracts and compounds from E. spiralis

Conc (µg/mL)	% inhibition				
	QC	Em	Ec	Ep	C1
500	99.12 ± 4.12	90.10 ± 3.41	80.26 ± 7..23	75.46 ± 8.14	91.32 ± 4.56
250	90.74 ± 3.98	83.61 ± 2.89	65.86 ± 3.09	60.24 ± 5.36	87.59 ± 4.23
125	79.95 ± 4.19	73.58 ± 5.02	58.42 ± 4.71	52.29 ± 3.76	80.18 ± 5.95
62.5	70.68 ± 0.75	65.12 ± 6.21	50.89 ±5.11	50.02 ± 4.98	74.24 ± 3.51
31.25	60.91 ± 3.67	48.37 ± 4.09	48.81 ± 3.84	47.30 ± 4.23	58.46 ± 2.34
15.625	55.23 ± 2.61	40.88 ± 3.38	45.11 ± 9.08	44.56 ± 6.33	55.32 ± 3.98
7.8125	40.76 ± 0.21	35.34 ± 2.19	38.82 ± 6.75	28.64 ± 6.32	40.39 ± 4.10
IC_{50} (µg/mL)	18.15 ± 0.15	20.63 ± 0.44	74.96 ± 24.77	172.93 ± 1.77	19.98 ± 1.23

Values are expressed as mean ± SEM of triplicate measurement. Results were analysed using one-way ANOVA. Significant difference was determined using Tukey's HSD (at $p < 0.05$). QC (quercetin), C1 (3,4′,5,7-tetrahydroxyflavone), Em (*E. spiralis* methanol extract), Ec (*E. spiralis* chloroform extract), Ep (*E. spiralis* petroleum ether extract), IC_{50} (concentration of a sample required to inhibit 50% of the enzyme)

Inhibition of this enzyme is known to be one of the effective strategies in reducing and preventing postprandial hyperglycaemia in diabetes patients. α-Glucosidase inhibitory activities of the extracts as well as the isolated constituent from the active fraction are shown in Table 4. Compound 1 exhibited a remarkable inhibitory activity with low IC_{50} value of 19.98 ± 1.23 µg/mL. The inhibitory activity of QC was significantly higher ($p < 0.05$) in all concentrations, although there was no significant difference between its IC_{50} and those of C1 and Em. Ep showed the lowest inhibition value while Em and Ec were considered active, having their IC_{50} less than 100 µg/mL. Meanwhile, Em, which had the least IC_{50} (20.63 ± 0.44 µg/mL) among these extracts, was considered as the most potent α-glucosidase inhibitor. Strong inhibitory activities of methanol extracts from various plants have previously been reported (Gholamhosenian & Fallah, 2009; Ortiz et al., 2007). The low digestive enzyme-inhibitory activity of Ep may be due to the presence of biologically active phytochemicals in an insignificant amount.

CONCLUSION

This present study has showed that *E. spiralis* stem bark possesses a strong α-glucosidase inhibitory and antioxidant activities. It acts by scavenging free radicals and inhibiting α-glucosidase enzyme in a dose-dependent manner. Moreover, methanol extract had the highest phenolic and flavonoids contents among all the extracts and subsequently exerted the highest antioxidant and α-glucosidase inhibitory activities. Fractionation and purification of this extract led to isolation of a bioactive compound, which was characterized as 3,4′,5,7-tetrahydroxyflavone and also known as kaempferol. The presence of 3,4′,5,7-tetrahydroxyflavone in the stem bark of *E. spiralis* as an antioxidant and α-glucosidase inhibitor is being reported for the first time. This plant may therefore have potential therapeutic applications for diabetes mellitus type 2 management.

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Protease Inhibitory Activity and Protein Analysis of Catfish (*Pangasius hypopthalmus*) and Swamp Eel (*Monopterus albus*) Blood Plasma

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ABSTRACT

Protease inhibitors can prevent protein from degradation caused by protease activity. Blood plasma contains a variety of protease inhibitors. The objective of this study was to investigate the potential use of crude blood plasma from catfish *(Pangasius hypopthalmus*) and swamp eel (*Monopterus albus*) as protease inhibitors. The parameters observed were moisture content, protein content, ash content, inhibitory activity to trypsin and papain enzymes and protein profile of blood plasma. The inhibitory activity increased as the volume of blood plasma increased (25 µL, 50 µL, 75 µL, 100 µL). The inhibitory activity of blood plasma from catfish was 7.66-50.73% to trypsin enzyme and 20.34-83.05% to papain enzyme while the inhibitory activity of blood plasma from swamp eel was 9.49-46.35% to trypsin enzyme and 28.81-64.41% to papain enzyme. The highest inhibitory activity was demonstrated by swamp eel blood plasma with molecular weight between 19.84-174.14 kDa (13 proteins). Protein content of blood plasma from swamp eel (11.92%) was lower than catfish (16.63%) but resulted higher enzyme inhibitory activity. Further research can be conducted to carry out purification steps on blood plasma that are expected to show better inhibitory activity.

Keywords: Blood plasma, catfish, protease inhibitor, swamp eel

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INTRODUCTION

Fish is a commonly used food around the world and is classified as perishable food. One of the factors that affect the rapid change in fish quality is the high protein content. In general, protein content in fish is 15-20%, but protein content lower than 15% and higher than 28% is also found in some fish species (Murray & Burt, 1983). Protease enzymes act on fish protein to bring about its deterioration in its quality.

The decline in quality after the post mortem phase is one of the most unfavourable changes in fish muscle. During post mortem, degradation of muscle proteins contributes to the rapid softening of flesh. The protein proteolysis can be attributed to endogenous protease activity (Chéret et al., 2007). Two characterized proteolytics are known to hydrolyze protein during post mortem storage of meat and fish muscle: calpains and cathepsins (Jiang, 2000; Ouali, 1992). Cathepsin L is one of the most important protease enzymes in the softening process in fish meat, and in surimi processing it is a problem because washing cannot eliminate it. The presence cathepsin enzyme in surimi or fish mince results in a decrease in gel strength with a brittle and non-elastic gel at temperature around 60^{o}C (Rawdkuen et al., 2007b). Ho et al. (2000) reported that cathepsin L and and L-like in mackerel surimi had Myosin Heavy Chain (MHC)-degrading ability which consequently caused gel softening during setting at 40-45^{o}C. In addition, texture softening also occurs in grass carp fillets caused by Cathepsin B and L as the major endogenous enzymes that leads to proteolytic degradation (Ge et al., 2014).

One way to inhibit proteolytic degradation by cathepsin enzymes is by using blood plasma. Blood plasma contains a variety of protease inhibitors, including α2-macroglobulin which is a protease inhibitor of some protease classes with its mechanism of action baits and traps (Barret, 1981). Many studies have been conducted using plasma to inhibit the work of cathepsin enzymes, including cow blood plasma (Kang & Lanier, 1999; Marquez-Alvarez et al., 2015), pig blood plasma (Benjakul et al., 2001; Benjakul & Visessanguan, 2000), chicken blood plasma (Rawdkuen et al., 2007a) and salmon blood plasma (Fowler & Park, 2015). However, the utilization of blood plasma from cattle and chickens is limited due to mad cow disease infection as well as avian influenza in poultry.

Blood plasma comprises 46-63% of total blood volume, with moisture content being 92% (Martini, 2005). According to the Food and Agriculture Organization (Nomura, 2007), the global fish catch and aquaculture amounted to 106 million tons in 2004, and since blood is about 7% of body weight (Philips & Williams, 2011), this would amount to 7 million tons. Fish blood is generally not utilized but has the potential to be collected and processed to produce value-added foods based on its nutritional value and functional properties (Lynch et al., 2017). Fish blood can be extracted for application in the food, biomedical and pharmaceutical sectors (Lafarga et al., 2015; Lafarga et al., 2016; Mullen et al., 2015).

The objective of this study was to investigate the ability of blood plasma from two freshwater tropical fish (catfish and swamp eel) to function as protease inhibitor towards the enzymes trypsin and papain.

MATERIAL AND METHODS

Materials

Live catfish *(Pangasius hypopthalmus*) (size 2-4/kg) and swamp eel (*Monopterus albus*) (size 10-15/kg) were obtained from Indralaya traditional market, South Sumatera, Indonesia. Trypsin (from bovine pancreas), papain (from papaya latex), N_{α}-Benzoyl-$_{L}$-arginine-$_{DL}$ β-naphthylamide (BANA) and N_{α}-Benzoyl-$_{L}$-arginine 4-nitroanilide hydrochloride (BAPNA) were purchased from Sigma Aldrich (USA).

Collecting of Blood Plasma

The collecting of crude blood plasma was according to methods described in Fowler and Park (2015). Whole blood was collected from bleeding fish into bottles containing EDTA (Eethylenediaminetetraacetic acid), and then centrifuged for 15 min at 1500 g at 4^{o}C. The supernatant was regarded as plasma and kept in freezer until it was used.

Proximate Analysis

Protein Content (Bradford, 1976). The protein analysis followed the method of Bradford (1976) and the Bovine Serum Albumin (BSA) was used as the standard.

***Preparation of Bradford Reagent Solution*.** A 25 mL aliquot of ethanol (95%) was mixed with 5 mg of Commassie Brilliant Blue G250. The solution was then added to 50 mL H_3PO_3 85% and homogenized. The mixture was adjusted with distilled water until it reached a volume of 100 mL and kept at 4^{o}C before use.

***Preparation of Standard Solution*.** The preparation of standard solutions for protein analysis is given in Table 1. Ten mg of BSA was added to 10 mL of distilled water and then stirred with a magnetic stirrer to give a homogeneous solution. Specific volumes of BSA (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mL) were added to different volume of distilled water (0.9, 0.8, 0.6, 0.4, 0.2 and 0 mL) respectively, and then 5 mL of Bradford solution was added to each mixture. The mixtures were allowed to react for 30 minutes at room temperature. The absorbance was read at 595 nm.

Table 1
The concentration of standard solution (Bovine Serum Albumin)

BSA (mL)	Distilled water (mL)	Concentration (mg/mL)
0.1	0.9	0.1
0.2	0.8	0.2
0.4	0.6	0.4
0.6	0.4	0.6
0.8	0.2	0.8
1	0	1

***Preparation of Blank*.** One mL of distilled water was mixed with 5 mL of Bradford solution which was then homogenized and allowed to react for 30 minutes at room temperature. The absorbance was read at 595 nm.

Sample Assay. A 1 mL of sample plasma was mixed with 9 mL of distilled water. A 0.5 mL of the mixture was then added to 4.5 mL of Bradford reagent. The solution was allowed to react at room temperature for 30 minutes. The absorbance was read at 595 nm. The concentration of protein was determined by the following equation:

y = ax+b
y = absorbance of sample
a = slope
b = intercept
x = protein concentration of sample

Moisture Content. The moisture content was determined using the evaporation principle until the sample reached a constant weight at 105°C (Association of Official Analytical Chemists [AOAC], 2005). The blood plasma sample used was 1 mL.

Ash Content. The ash content analysis was determined by the combustion of organic compounds at 550°C (AOAC, 2005). The blood plasma sample used was 1 mL.

Trypsin Inhibition Assay. Trypsin inhibition was determined according to the methods of Fowler and Park (2015). Four different blood plasma volumes ((25, 50, 75, 100 µL) (Table 2) were diluted with distilled water and adjusted to 0.2 mL. 150 µL of the inhibitor solution was added to 300 µL of trypsin enzyme (20 µg/mL) and 150 µL of distilled water and pre-incubated at 37°C for 10 min. 750 µL of 0.4 mg/mL BAPNA in 50 mM tris-HCl buffer (pH 8.2) containing 20 mM $CaCl_2$ and pre-warmed to 37°C was than added, and the reaction mixture was incubated for 37°C for 10 minutes. The reaction was stopped by adding 150 µL of 30% acetic acid (v/v). Absorbance was read at 410 nm and inhibitory activity was expressed as percent decrease in OD_{410} compared to the control.

Papain Inhibition Assay. Papain inhibition was determined according to the method of Fowler and Park (2015). Four different blood plasma volumes ((25, 50, 75, 100 µL) (Table 2) were diluted with distilled water and

Table 2
Volume of catfish and swamp eel blood plasma sample for trypsin and papain inhibition assay

Sample	Papain Assay			Trypsin Assay		
	Plasma Volume (µL)	Protein Concentration (mg/mL)	Plasma Concentration (mg/mL)	Plasma Volume (µL)	Protein Concentration (mg/mL)	Plasma Concentration (mg/mL)
Catfish Plasma	25	0.21	1.25	25	2.08	12.5
	50	0.42	2.5	50	4.16	25
	75	0.62	3.75	75	6.24	37.5
	100	0.83	5	100	8.32	50
Swamp eel Plasma	25	0.15	1.25	25	1.49	12.5
	50	0.30	2.5	50	2.98	25
	75	0.45	3.75	75	4.47	37.5
	100	0.60	5	100	5.96	50

adjusted to 2 mL. 2 mL of 0.25 M sodium phosphate buffer (pH 6.0) containing 2.5 mM EDTA and 25 mM β-mercaptoethanol (βME) was added to 0.1 mL of papain solution (100 µg/mL) containing 25 mM sodium phosphate buffer (pH 7.0) and 2 mL of inhibitor solution. After preincubation at 37°C for 5min, 0.2 mL of 2 mM BANA was added to initiate the reaction. After 10 min of incubation, 1 mL of cold 2% HCl in ethanol was added to stop the reaction. 1 mL of 0.06% ρ-dimethylamino-cinnamaldehyde dye was then added to show the colour of the final solution. Absorbance was read at 540 nm and the inhibitory activity was expressed as the percent decrease in OD_{540} compared to the control.

Molecular Weight of Inhibitor

The SDS-PAGE procedure was followed according to the method described by Laemmli (1970). A 12% resolving gel and a 3.5% stacking gel were used. The sample buffer consisted of 188 mM M Tris-HCl pH 6.8, 15% β-mercaptoethanol, 3% SDS, 0.01% bromophenol blue, and 30% glycerol in deionized water. Sample preparation was conducted by mixing protein samples with loading buffer in a ratio 1:1 and heating the mixture to 95°C for 10 min to denature the protein samples. An SDS-PAGE broad-range molecular weight standard (6.5 to 200 kDa) (Bio-Rad, Alfred Nobel Drive, Hercules, CA, U.S.A) was used. The running buffer consisted of 0.12 M Tris base, 0.95 M glycine and 0.5% SDS (w/v) in deionized water. Samples and protein standard were run into well. After finishing the process, gels were then immersed in deionized water for 5 min and stained with coomassie blue stain buffer for 20 minuntes and destained with a destaining buffer for 1 min. The gels were then immersed in 1% acetic acid and incubated for 1 h on a shaker. The acetic acid was replenished every hour until the gel was completely de-stained.

Statistical Analysis

Only descriptive statistics was utilized for the reporting of the data.

RESULTS AND DISCUSSION

Proximate Analysis of Blood Plasma

Table 3 shows the proximate analysis for each blood plasma. The moisture content of catfish blood plasma was 91.46% and 93.05% for the swamp eel.

Table 3
The proximate analysis of catfish (Pangasius hypopthalmus) *and swamp eel* (Monopterus albus) *blood plasma*

Source of plasma	Proximate Analysis		
	Moisture content (%)	Protein (mg/mL)	Ash (%)
Cat Fish	91.46	16.63	0.75
Swamp eel	93.05	11.92	0.92

According to Kisia (2016) and Martini (2005), moisture content in plasma of vertebrates is 90-92%. The difference of moisture content in plasma is due to differences in the chemical composition, such as protein content and ash content, which affects moisture content. Proteins are important components and functions in maintaining water balance in the blood and

tissues, regulating blood volume, helping the transport of fat, vitamins, and hormones and as antibodies.

The protein content of catfish plasma was 16.63 mg/mL and the swamp eel plasma was 11.92 mg/mL. Protein in plasma reflects the blood protein content, except for hemoglobin. Plasma proteins are divided into three main components i.e. albumin (42% w / v), globulin (56%) (α1-globulin, α2-globulin, β-globulin, and γ-globulin) and fibrinogen (1%). The largest proportions contained in solutes include proteins, which account for 6% -8% in blood plasma (Moure et al., 2003). Kisia (2016) suggested that solutes in fish blood plasma varied between freshwater fish and marine fish. The protein content of fish plasma is realtively low when compared to other vertebrates. The blood proteins are important in the maintenance of the right osmotic pressure and viscosity of blood.

The ash content of catfish and swamp eel plasma were 0.75% and 0.92% respectively. The difference of the ash content in plasma is related to endogenous (genetically controlled and associated with species-specific life cycles) and exogenous (such as environmental and dietary) factors (Perschbacher & Stickney, 2017). Ash content reflects the mineral content in blood plasma. Minerals in the blood plasma are part of a minor component of blood plasma which amounts to 1% (Martini, 2005; Moure et al., 2003). Minerals make up the normal extracellular fluid ion composition for vital cellular activity and contributes to the osmotic pressure of body fluids. The major plasma electrolytes are Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, HCO^{3-}, HPO_4^{2-}, and SO_4^{2-} (Martini, 2005).

Inhibitory Activity of Blood Plasma

The inhibitory activity of blood plasma to protease enzyme increased as the concentration of blood plasma for both fishes increased. The protease inhibitory activity ranged from 7.66 to 50.73% for trypsin and 20.34 to 83.05% for papain for the catfish blood plasma (Figure 1), while the protease inhibitory activity of the swamp eel blood plasma ranged from 9.49 to 46.35% for trypsin and 28.81 to 64.41% for papain (Figure 2).

The blood plasma protease inhibitory activity to papain was higher than that of trypsin. This result was in contrast with the recommendation of Yongswatdigul et al. (2014) and Sriket (2014), where the surimi made from tropical fish were susceptible to serine protease attacks that caused protein degradation. But this was in line with the result of Ge et al., (2014) that reported softening occured in grass carp fillets caused by cathepsin B and L as the major endogenous enzymes leading to proteolytic degradation.

The highest protease inhibitory activity on trypsin and papain was by the swamp eel blood plasma even though its protein concentration was lower than that of the catfish blood plasma (0.15-0.60 mg/mL and 0.21-0.83 mg/mL, respectively). This was probably due to the higher efficacy of the protein plasma of the eel acting as a protease inhibitor as compared to that in

the catfish. Fowler and Park (2015) showed that protease inhibitory activity of salmon plasma ranged between 25% - 81% for papain and below 20% for trypsin. The higher inhibitory activity of the salmon blood plasma as compared to that of the catfish and swamp eel (this study) was probably related to it being free of impurities such as water, fat, ash, and other non-protein components.

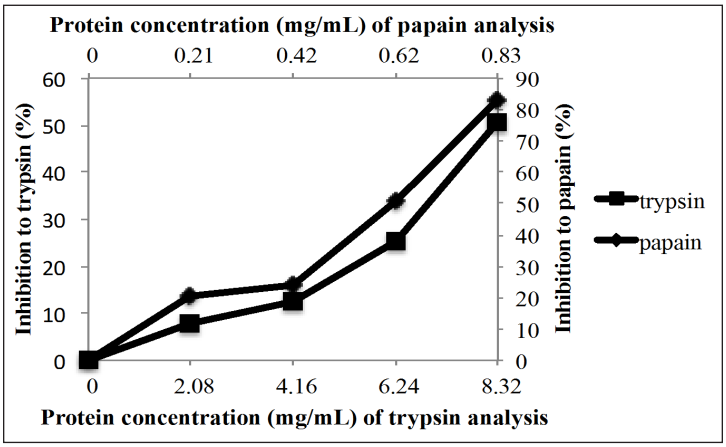

Figure 1. Inhibitory activity of catfish (*Pangasius hypopthalmus*) blood plasma to trypsin and papain enzymes

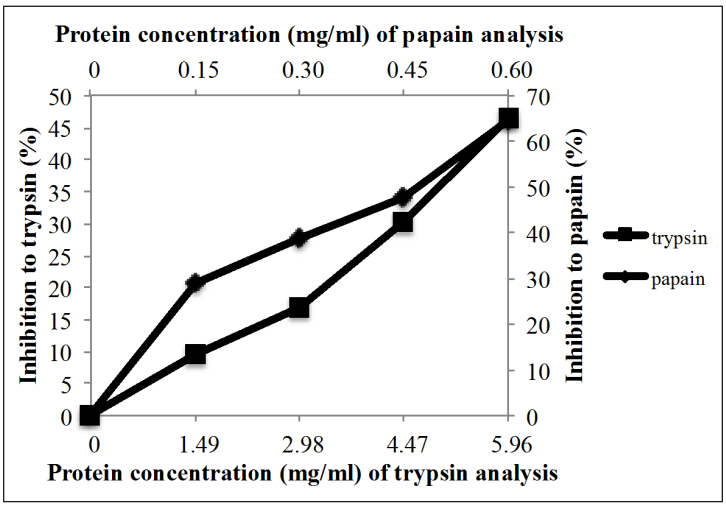

Figure 2. Inhibitory activity of swamp eel (*Monopterus albus*) blood plasma to trypsin and papain enzymes

Molecular Weight of Blood Plasma

The molecular weight of catfish (C1-C3) and swamp eel blood plasma (S1-S3) is in the range of 9.64 to 124.36 kDa (10 proteins) and 19.84 to 174.14 kDa (13 proteins) respectively (Figure 3). The molecular weight of the protein bands detected in the blood plasma of the catfish and swamp eel (this study) showed similarities with

molecular weight of pig plasma. The band is presumed to be α-globulin protein to have molecular weight ranging from 105 kDa - 150 kDa, while albumins had molecular weight of 65 kDa - 69 kDa. α_2-globulin is a protease inhibitor found indigenously in blood plasma and shows inhibitory activity againts serine, cysteine, carboxyl and metallo-proteinases (Benjakul et al., 2001; Lee e al., 2000). According to Benjakul and Visessanguan (2000), the serine protease inhibitor is in the range of 58-64 kDa and this is within the range of the proteins in catfish and eel plasma which is able to inhibit the enzymes chymotrypsin and trypsin. Li et al. (2008) reported the presence of the cysteine protease inhibitor at 55 kDa. The variation of the proteins from the catfish and swamp eel determined by the SDS-PAGE analysis reflected their inhibitory activity. The higher variation of the proteins in the swamp eel blood plasma may explain its higher inhibitory activity than that of the catfish.

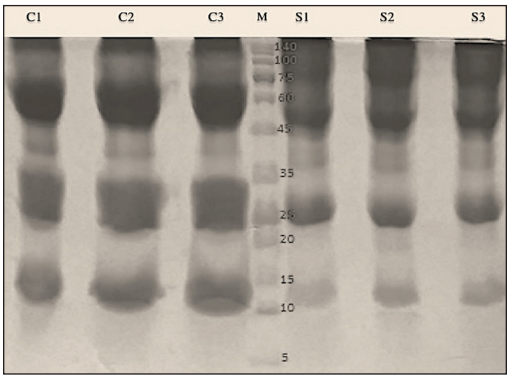

Figure 3. Molecular weight of catfish (*Pangasius hypopthalmus*) (C1, C2, C3) and swamp eel (*Monopterus albus*) (S1, S2, S3) blood plasma (M= protein marker)

CONCLUSION

Both the catfish and swamp eel blood plasma showed inhibitory activity towards trypsin and papain. Papain is a cysteine protease and its inhibition was higher than that of trypsin. The highest inhibitory activity to protease enzyme was from the swamp eel blood plasma.

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Microbial Contamination in Urban Tropical Lentic Waterbodies and Ponds along an Urbanization Gradient

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ABSTRACT

This study aimed to investigate the seasonal variation of microbial quality in urban waterbodies along urbanization gradients. Bimonthly samples were collected from 14 recreational lakes and flood mitigation ponds in the Klang Valley, Malaysia, between May and October 2017. Samples were analysed for the presence and abundance of *Escherichia coli*, *Clostridium perfringens*, faecal coliforms (FC), faecal streptococci (FS), enterococci and total coliforms as indicator organisms, using standard methods. All studied lakes contained indicator bacteria that exceeded the National Lake Water Quality Standards (NLWQS) to varying degrees. The mean of the FC/FS ratios in all lakes exceeded four, indicating that the faecal contamination might have originated from human sources. *Escherichia coli*, *C. perfringens* and faecal coliform concentrations were negatively correlated with temperature ($P < 0.01$) and positively correlated with turbidity and suspended solids ($P < 0.05$). Non-parametric test results revealed that only the density of *C. perfringens* varied significantly according to season and urbanization impacts ($P < 0.05$). The Secchi depth transparency and dissolved oxygen (DO) levels explain the largest variation in bacterial communities. This study showed that contamination of faecal bacteria in the waterbodies varied spatially and temporally along urbanization gradients. Water quality monitoring and improvements are needed before the waterbodies can be used for direct body-contact recreation and as alternative water sources for drinking purposes.

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INTRODUCTION

Small waterbodies, including flood mitigation ponds and ex-mining pools, are important alternative water sources during drought. Due to their urban positioning, much of the area around these ponds has been converted to recreation parks worldwide, thus the waterbodies are used for primary or secondary recreational purposes. Converting ex-mining pools to water storage supply and recreational facilities has raised concerns about the impact of water quality on humans, in particular, the heavy-metals content (Kusin et al., 2016). Additionally, the presence of pathogenic bacteria such as *E. coli* and faecal enterococci can cause various water-borne diseases on contact with humans. The type of disease is dependent on the species involved. Examples are diarrhoea (*E. coli*), leptospirosis (*Leptospira* spp.), Cryptosporidiosis (*Cryptosporidium* spp.) and cholera (*Vibrio cholerae*) (Hipsey & Brookes, 2013; World Health Organization [WHO], 2003). All these water-borne diseases induce abdominal pain, diarrhoea and vomiting (WHO, 2003). Despite the increasing number of studies on water quality undertaken in developing countries, studies involving microbiological analyses in countries such as Malaysia are limited to biochemical oxygen demand (BOD), chemical oxygen demand (COD), and faecal coliform and total coliform studies (Department of Environment [DOE], 2016; Said et al., 2012a, 2012b). In developed countries, such as the United States and those in Europe, pathogenic bacteria, protozoa and viruses are widely monitored as water quality parameters. However, such microbiological assessment is not commonly undertaken in developing countries due to limited expertise, a lack of facilities and budget restraints on the complex and costly analytical testing procedures required for microbial detection. The faecal contamination of urban waterbodies remains a problem for many of the developing countries in Asia and Africa, due to lack of sanitation, and this leads to continued faeces-related diseases such as cholera and diarrhoea (Demanou & Brummett, 2003; Henny & Meutia, 2014; Komarulzaman et al., 2017).

A new standard for lake water was introduced by the National Hydraulic Research Institute of Malaysia in 2015 and approved for application in Malaysia in 2017. In addition to BOD, COD and total coliforms, the National Lake Water Quality Criteria and Standards (NLWQS) recommended the use of *Escherichia coli*, enterococci and *Clostridium perfringens* as bacterial indicators for the ambient water quality of stagnant water (National Hydraulic Research Institute of Malaysia [NAHRIM], 2015; Sharip & Suratman, 2017). All these faecal bacterial indicators are present in human faeces, in the order *C. perfringens* < enterococci < *E. coli* (Edberg et al., 2000). Their survival rates in the

environment, are in the order *E. coli* (weeks to a month) < enterococci (weeks) < *C. perfringens* (months to years) (Edberg et al., 2000). Little is known about the occurrence of most of these faecal bacterial indicators in the waterbodies in Malaysia, making informed decision-making for effective management difficult.

Few microbial studies have been carried out in waterbodies that are used for public consumption, in particular, in those that meet recreational needs. This includes the occurrence of protozoa, namely, *Giardia* and *Cryptosporidium* oocysts, which have been studied in rivers and recreational lakes in the Malaysian Peninsular (Jali & Ithoi, 2009; Lee et al., 2014; Onichandran et al., 2013). These studies frequently detect *Giardia* rather than *Cryptosporidium* in rivers, with more oocysts found in the downstream part of the river followed by midstream and upstream sections (Jali & Ithoi, 2009; Lee et al., 2014). In another study, conducted in two recreational waterbodies, *Cryptosporidium* spp., *Giardia* spp., *Ascaris* spp., *Acanthamoeba* spp. and hookworm were detected in both recreational lakes, while *Schistosoma* spp. were found in only one lake (Onichandran et al., 2013). A study carried out by Hamzah and Hattasrul (2007) in Chini Lake in Malaysia, in 2005, reported faecal contamination, namely, total coliforms and *E. coli*, in the range 10^2 to 10^5 in some parts of the lake, indicating that the lake was unsuitable for recreational use. The occurrence of faecal bacteria in water in temperate countries has been associated with physical factors such as temperature, solar radiation and salinity that influence their survival rates (Hughes, 2003; Sadowsky & Whitman, 2011; Whitman et al., 2004). For example, the higher the solar irradiance and salinity levels, the lower the survival rates of faecal coliforms in the water (Sadowsky & Whitman, 2011; Whitman et al., 2004). Elevated levels of *E. coli* in lake water were detected during the wet season or after rainfall events due to surface run-off while higher levels of *E. coli* in the near-shore water was linked to resuspension of beach sand driven by onshore winds (Isobe et al., 2004; Whitman et al., 2006). The urbanization process could also shape the microbial density: higher densities of *E. coli* were found at sites near residential and commercial areas compared to other land uses in the upper Blackstone River watershed, USA (Wu et al., 2011).

The present study aimed at investigating the occurrence of six faecal indicator bacteria (*C. perfringens*, total coliforms, faecal coliforms, faecal streptococci, *E. coli* and enterococci) in urban tropical lentic waterbodies and how their abundance correlates with environmental factors and the level of urbanization. This study also aimed to provide baseline data on the faecal indicator bacteria occurrence, in line with the application of the national lake water quality standard throughout the country to support effective management of lakes and ponds.

MATERIALS AND METHODS

Study Site

This work focused on 14 ponds of varying sizes, types and shapes, as shown in Figure 1 and Table 1. The waterbodies included five recreational lakes and nine flood-retention ponds that had recently been identified as potential sites for conjunctive water supply systems for greater Kuala Lumpur. Two ponds (L4 and L7) received discharges from nearby wastewater treatment facilities.

Field Measurement

In situ field measurements were carried out in triplicates on all ponds and lakes. In situ measurements of total depth, dissolved oxygen (DO), pH, temperature, salinity and conductivity were recorded with an YSI multi-parameter probe (YSI Incorporated, Yellow Springs, OH) and Secchi depths (SD) were measured using a Secchi disk. All parameters in the multi-parameter probe were calibrated using standard procedures prior to sampling. Salinity was included to detect levels of chlorination in the water. Surface water samples were collected in 1-litre amber bottles, stored in cooler boxes at 4°C and sent to an accredited laboratory for microbial analysis using established and validated methods. Total coliform, faecal coliform, *E. coli*, enterococci and faecal streptococci were analysed using membrane filtration as described in the standard methods for the examination of water and wastewater (American Public Health Association [APHA], 2012). Total coliform was measured using the membrane filtration method, enterococci and faecal streptococci using membrane filter techniques and *E. coli* and faecal coliform using a thermotolerant membrane filter procedure. *C. perfringens* was analysed using the membrane filtration method (United Kingdom Environment Agency [UKEA], 2010). Chromocult Coliform Agar was used to grow *E. coli*, faecal coliforms and total coliforms at 35°C, over an incubation periods of two days. The media and incubation periods for growing faecal streptococci and enterococci and for growing *C. perfringens* were Kenner Faecal Agar (48 hours) and Tryptose Sulfite

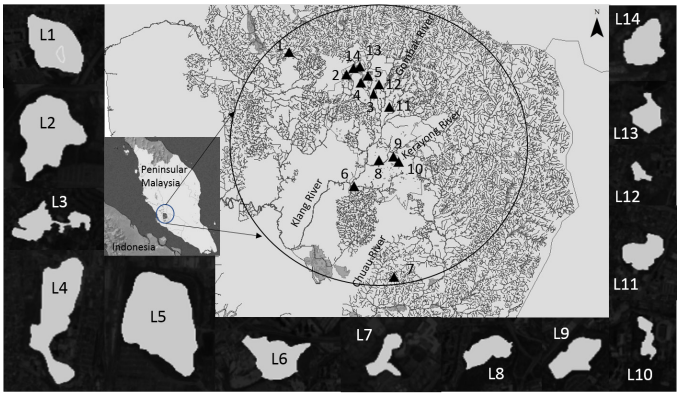


Figure 1. Map of the study urban lakes. Numbers refers to lake

Table 1

Physical characteristics of the studied lakes

Lake	Latitude and Longitude	Surface Area (km^2)	Types of Ponds	Ranging Depth
L1	101°31.009'E, 3°15.000'N	0.28	Water Recreational	0.3 – 1.7
L2	101°38.013'E, 3°13.008'N	0.42	Water Recreational	0.3 – 2.1
L3	101°42.006'E, 3°10.011'N	0.14	Water Recreational	0.6 – 3.7
L4	101°40.000'E, 3°12.013'N	0.40	Retention pond	1.4 – 5.0
L5	101°40.010'E, 3°13.006'N	0.65	Retention pond	1.7 – 29.0
L6	101°39.005'E, 3°3.0137'N	0.21	Retention pond	0.5 - 2.5
L7	101°42.007'E, 2°56.004'N	0.08	Retention pond	1.3 – 1.9
L8	101°41.009'E, 3°6.002'N	0.12	Retention pond	2.3 – 16.3
L9	101°42.013'E, 3°6.008'N	0.15	Retention pond	5.5 – 16.3
L10	101°43.003'E, 3°5.016'N	0.05	Water Recreational	0.6 – 2.2
L11	101°43.002'E, 3°12.004'N	0.07	Water Recreational	0.3 - 0.7
L12	101°43.003'E 3°11.003'N	0.02	Retention pond	0.9 – 1.0
L13	101°39.015'E, 3°14.003'N	0.09	Retention pond	0.3 – 0.4
L14	101°39.006'E, 3°13.016'N	0.16	Retention pond	0.3 – 2.1

Cycloserine Agar (24 hours) respectively. All bacterial colonies were calculated based on CFU calculations.

Additional water samples were taken in separate bottles: 100-ml bottles for the analysis of total phosphorus (TP), 500-ml bottles for the analysis of ammoniacal nitrogen, nitrate, iron, manganese and chemical oxygen demand (COD) and 1-litre bottles for the analysis of biochemical oxygen demand (BOD) and chlorophyll *a* (Chl *a*), in accordance with the standard method (APHA, 2012). *In situ* measurements of total depth, dissolved oxygen (DO), pH, temperature, salinity and conductivity were recorded with an YSI multi-parameter probe (YSI Incorporated, Yellow Springs, OH) and Secchi depths (SD) were measured

using a Secchi disk. Total suspended solids (TSS) concentrations were measured using a portable suspended solids meter (Lovibond, The Tintometer Ltd, Amesbury, UK). All measurements and sample collections were performed twice: during the dry months (16th May – 8th June) and the wet months (1st -15th October), at no fewer than two locations in each lake and pond.

Data Analysis

Clustering and non-metric multidimensional scaling (NMDS), based on the Bray-Curtis dissimilarity matrix (Clarke, 1993), were performed to visualize multivariate patterns among the indicator bacteria within the 14 lakes and ponds under study. The waterbodies included five recreational lakes and nine flood-retention ponds that had recently been identified as potential sites for conjunctive water supply systems for greater Kuala Lumpur. Principal component analysis (PCA) was initially applied to reduce the number of environmental variables and to summarize the pattern of correlations between total depth (maximum water depth), Secchi depth, temperature, pH, DO, turbidity, conductivity, BOD, COD, ammoniacal nitrogen, nitrate, ammonium, total suspended solids, surface area and shoreline length. Principal coordinates analysis (PCoA) was performed to correlate environmental variables with the ordination axes and to extract meaning from the axes produced in NMDS (Anderson & Willis, 2003). NMDS, PCA and PCoA calculations were based on lake averages.

Correlations between bacterial parameters and environmental variables were generated to assess the linear relationships between the parameters. Non-parametric multivariate multiple regression (DISTLM) was employed to model the relationship between the microbial parameters and the 15 environmental variables. Each variable was initially examined in a marginal test (excluding other variables) and then subjected to a forward selection procedure (Bayesian information criterion, BIC) with sequential tests. Significance was performed using 9,999 permutations of the residuals under the reduced model.

Following the scale described by Pawlikiewicz and Jurasz (2017), an urban impact assessment (UIA) was included as a factor in the analysis, in order to evaluate the impact of urbanization within a 1-km radius of the lake. The three-scale categories used were: 1 – high-density development and completely urbanized; 2 – mid-density development with significant urbanization and 3 – low-density urbanization area. Seasonal differences were also analysed using non-parametric Wilcoxon signed-rank tests and these were based on two categories: dry (May to September) and wet (October to December). Wilcoxon signed-rank tests were also used to determine any significant differences in microbial parameters between the ponds used for recreation and the flood-retention ponds. The linear relationships between individual variables were based on the Spearman correlation. Microbial data and other environmental variables were log transformed to improve normality prior to

multivariate analysis. Correlation and non-parametric Wilcoxon signed-rank tests were performed using SPSS 16.0 (SPSS Inc.) and multivariate methods (NMDS, PCA, PCoA and DISTLM) were conducted using PRIMER 6 and PERMANOVA+ (Plymouth Marine Laboratory, Plymouth, UK).

RESULTS

Water Quality and Bacterial Indicator Ratios

The temperature in the study lakes ranged between 26.7°C and 32.6°C, while the pH ranged between 7.35 and 8.51; all of which are within the NLWQS limits. Dissolved oxygen (DO) concentrations ranged between 4.91 and 10.8 mg/L, while TSS ranged between <0.1 and 366 mg/L. Three lakes (L6, L7 and L10) had DO values of less than 6.5 mg/L and lakes L9 and L13 had DO values exceeding 9.5 mg/L. The BOD and COD in these lakes were high. All BOD values exceeded the standard limit of 3 mg/L, with values ranging between 5.7 and 21.5 mg/L. The COD concentration ranged between 8.1 and 131.6 mg/L and only one lake (L7) had a COD level that complied with the NLWQS (<10 mg/L). The highest BOD and COD values were recorded in L14, followed by L6 and L13. Ammoniacal nitrogen was high in all lakes, ranging between 0.08 and 6.5 mg/L. High BOD and ammoniacal nitrogen levels have been associated with faecal contamination. Secchi depth transparencies were in the range <0.1 m to 2.1 m, while Chl *a* was in the range 1.7 to 86 µg/L. Based on biological productivity, one lake was categorized as oligotrophic, two lakes were mesotrophic, and the remainder were categorized as hypereutrophic. Nine waterbodies failed to meet the transparency criteria due to their low transparency (<0.6 m) and only two recreational lakes met the Chl *a* limit (10 µg/L). The TP values ranged between 0.05 and 1.1 mg/L and all of the samples exceeded the criteria of 0.01 mg/L and 0.035 mg/L for primary and secondary body contact.

Mean *E. coli* and *C. perfringens* contents were in the range 0.5 to 79,133 cfu100 ml^{-1} and 6.8 to 3,333 cfu100 ml^{-1}, respectively (Table 2). The highest *E. coli* and *C. perfringens* values were recorded in lake L6. Only L3 and L12 had *E. coli* of less than 100 cfu100 ml^{-1}. The faecal coliform and total coliform values in all lakes were in the ranges 700 to 60,900 cfu100 ml^{-1} and 2,425 to 205,000 cfu100 ml^{-1} respectively. The faecal coliform values in the samples taken from all the lakes exceeded the recommended 150 cfu100 ml^{-1} and the total coliform values in two lakes (L3 and L10) were below the recommended values for recreational purposes of 5000 cfu100 ml^{-1}. Enterococci and faecal streptococci in all samples were <1 cfu100 ml^{-1}.

An NMDS plot shows that the bacterial communities in the studied lakes are grouped into four distinct clusters for samples with more than 95% similarity (Figure 2). The hypereutrophic lakes (L9 and L13) form one cluster with 97.9% similarity, the cluster L11 and L7 has 96.7% similarity, the cluster L4 and L14 has 96.6% similarity and the cluster

L6 and L1 has 96.5% similarity. The cluster L3 and L12 with 93.1% similarity, has low *E. coli* and *C. perfringens content.* Cluster (L4, L5, L7, L11, L14) with 85% similarity contains high faecal and total coliforms. No distinct pattern was observed in the microbial quality of the clustered lakes in terms of trophic levels and lake use.

According to Gannon and Busse (1989), an FC/FS ratio of more than four is listed as originating from a human source, while a FC/FS ratio of <0.7 originates from animal sources. In this study, all samples had a FC/FS ratio of more than four, indicating that faecal contamination in the water originated from in human sources. The NLWQS recommended levels for *E. coli*, enterococci and faecal coliforms are 100 cfu100 ml^{-1}, 33 cfu100 ml^{-1} and 150 cfu100 ml^{-1}, respectively. This yield an EC/FC (*E. coli*/faecal coliforms) ratio of 0.67. The mean EC/FC ratio in all lakes was in the range 0 to 1.34; only two ponds (L14 and L15) were well above the ratio of 0.67 set by NLWQS, while the others were within the recommended values.

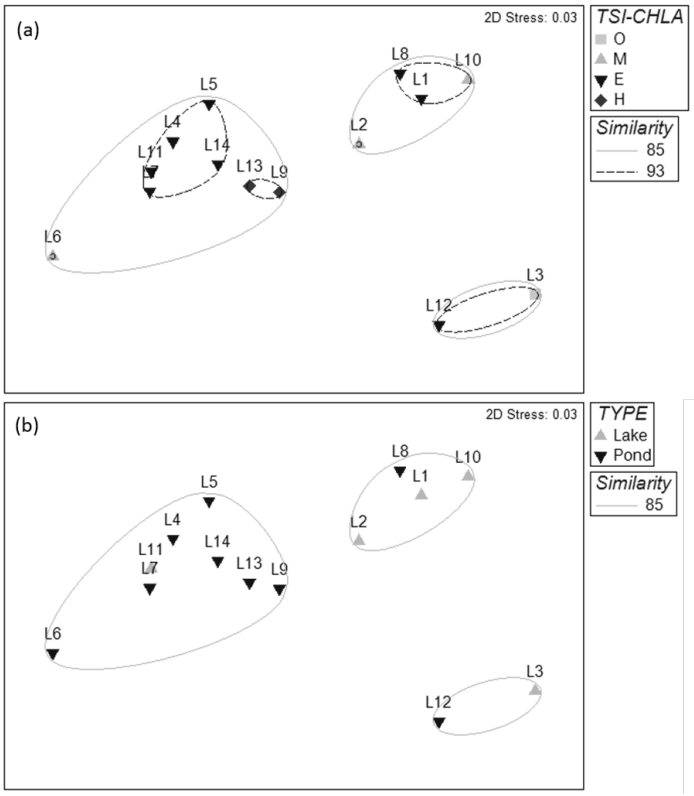


Figure 2. NMDS plots of microbial quality in urban lakes with (a) trophic levels and (b) lake type. O – oligotrophy; M – mesotrophy; E – eutrophy; H – Hyper-eutrophy

Table 2

Mean values (±SE) of microbiological parameters in the studied lakes

Lake	Clostridium perfringens	*Escherichia coli*	faecal coliform	Total Coliform	Enterococci	Trophic Status
			cfu 100 ml^{-1}			
L1	7±4	116±50	1200±491	5920±3331	<1	Eutrophic
L2	17±11	147±77	1725±880	25350±13561	<1	Mesotrophic
L3	34±34	0.5±0	700±700	3250±1350	<1	Oligotrophic
L4	40±47	7050±5013	15725±6848	96350±54689	<1	Eutrophic
L5	8±6	1500±463	14467±6018	89350±41884	<1	Eutrophic
L6	3333±1241	79133±72940	60867±56575	60167±52934	<1	Mesotrophic
L7	276±148	3367±953	20117±15055	204667±189431	<1	Eutrophic
L8	6±3	45±22	2680±1845	10080±5784	<1	Eutrophic
L9	94±56	550±289	2540±498	39400±20611	<1	Hyper-eutrophic
L10	6±3	110±64	825±284	2425±487	<1	mesotrophic
L11	79±39	3067±593	50833±29311	228333±13146	<1	Eutrophic
L12	45±32	0.5±0	850±177	31200±14707	<1	Eutrophic
L13	63±30	1175±683	3425±856	42700±11066	<1	Hyper-eutrophic
L14	37.3±13	2964±1876	6900±1804	59267±32879	<1	Eutrophic
NLWQS	absent	100 (600)	150 (1000)	5000	33	

Note. Values in NLWQS refers to the Category A (Primary body contact) of the standard, items in bracket represents Category B (Secondary body contact) of NLWQS

Relationship between Bacterial Quality, Water Quality and Urbanization Impacts

The correlations between bacterial parameters and the environmental variables are shown in Table 3. All *E. coli*, *C. perfringens* and faecal coliforms were negatively correlated with temperature ($P < 0.01$) and positively correlated with turbidity ($P < 0.05$). In terms of nutrients, *E. coli*, faecal and total coliforms were positively correlated with TP. All four bacterial parameters were positively correlated with TSS ($P < 0.05$) and negatively correlated with Secchi depth transparency ($P < 0.05$). No correlation was observed between microbial quality and surface area or shoreline length. Dissolved oxygen (DO) and pH levels were significantly correlated with temperature ($r = 0.736$ and $r = 0.701$). NMDS test results indicated that only *C. perfringens* significantly differed between seasons ($P = 0.039$) and according to urbanization impact ($P = 0.033$) (Figure 3a & 3b). Other than L9, where the mean values were higher than in the dry season, *C. perfringens* was not detected in any of the lakes during the wet season. There was little variation in the mean *E. coli* concentration between the dry and wet seasons, but *E. coli* values were higher in some waterbodies during the wet season (L5, L14).

DISTLM showed that seven parameters explained the variation within the bacterial community. Secchi depth, DO and TP varied significantly between lakes. The results of DISTLM tests (Table 4) revealed a positive match between the environmental variables and FIB. Secchi depth explained the largest contribution (~49.4%) of the variation in the microbial composition in all datasets. This was followed by DO and TP concentrations, contributing a further 19.1% and 6.3% of the variance, respectively. Taken together, other parameters, such as TSS, manganese, chlorophyll-*a* and salinity, explained about 16% of the variance. Figure 4 illustrates the PCoA ordering of the bacterial values and environmental data.

Table 3

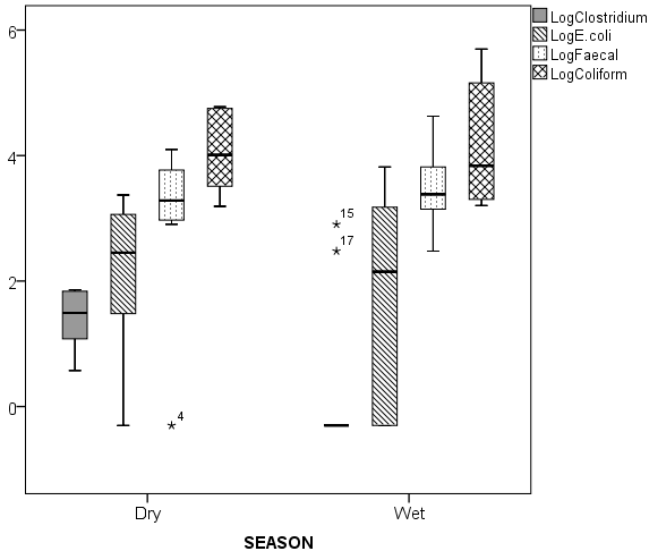
Correlation between bacterial concentrations and physico-chemical parameters

	Clostridium perfringens	*Escherichia coli*	faecal coliform	Total Coliform
Total depth	-0.209	-0.024	0.046	-0.088
SD	-0.659*	-0.641*	-0.718**	-0.711**
Temperature	-0.402	-0.719**	-0.635*	-0.503
pH	0.002	-0.385	-0.297	-0.275
DO	-0.178	-0.530	-0.367	-0.200

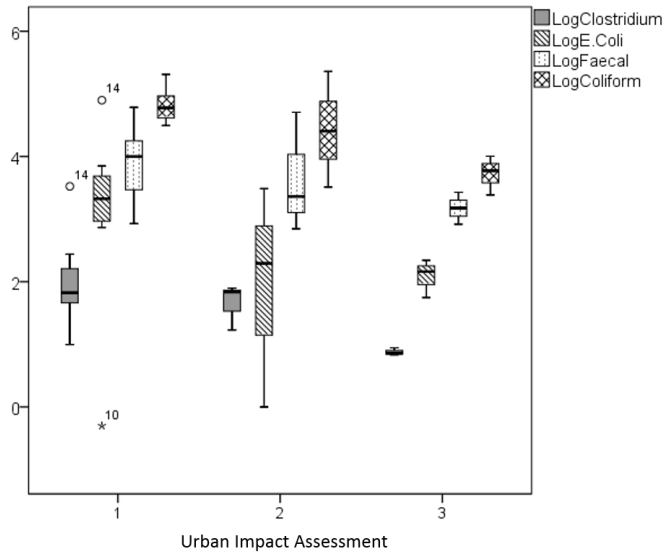
Table 3 *(Continue)*

	Clostridium perfringens	*Escherichia* coli	faecal coliform	Total Coliform
Conductivity	0.458	0.407	0.310	0.123
BOD	0.525	0.349	0.327	0.407
COD	0.284	0.209	0.055	-0.051
TP	0.301	0.587*	0.653*	0.560*
AN	0.279	0.481	0.420	0.363
NO_3	0.339	-0.088	-0.175	-0.356
Chl *a*	0.196	0.248	0.336	0.477
Turbidity	0.653*	0.552*	0.560*	0.578**
TSS	0.764**	0.577*	0.621*	0.632*
Surface area	-0.099	0.244	0.200	0.112
Shore length	0.046	0.231	0.108	0.068
C. perfringens	1	0.582*	0.530	0.574*
E. Coli	0.582*	1	0.895**	0.815**
faecal coliform	0.530	0.895**	1	0.908**
Total coliform	0.574*	0.815**	0.908**	1

Note. * = $P < 0.05$, ** = $P < 0.01$



3(a)



3(b)

Figure 3(a) & 3(b). Boxplot of microbial quality with seasons and urban impacts

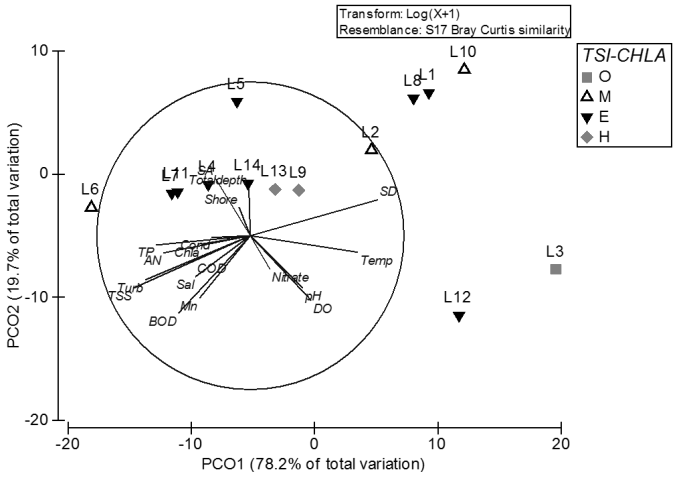


Figure 4. PCoA plots of microbial quality with other environmental variables. O – oligotrophy; M – mesotrophy; E – eutrophy; H – Hyper-eutrophy

Table 4

Results of the DISTLM analysis of the dataset of the fourteen lakes

Sequential Tests					
Variable	BIC	Pseudo-F	P	% Var	% Cum.
SD	65.375	11.73	0.001**	49.43	49.43
DO	61.368	6.6828	0.002**	19.11	68.54
TSS	60.273	3.0566	0.062	7.36	75.91
Sal	59.887	2.1712	0.147	4.68	80.59
TP	57.058	3.8226	0.043*	6.28	86.87
Manganese	56.363	1.8823	0.202	2.78	89.65

Note. BIC Bayesian Information Criterion, %Var percentage of variance in species data explained, % Cum cumulative percentage of variance explained *= $P < 0.05$, **= $P < 0.01$

DISCUSSION

This study shows that urban lakes and ponds in the Klang Valley have high bacterial loads. *C. perfringens*, *E. coli*, total coliform and faecal coliform presence in varying concentrations was observed in all lakes, and most lakes exceeded the national lake water quality standard for recreational purposes. Only enterococci and faecal streptococci were not detected in all samples throughout the study period. The bacterial indicator ratio indicated that most FIB was human in origin. *Escherichia coli* is known as a principal facultative aerobic bacterium found in the intestinal tracts of humans and animals, and the content in faeces varies among host species. The *E. coli* content per weight in human faeces and those of domestic animals ranges between 10^7 and 10^9 and between 10^4 and 10^6, respectively (Tenaillon et al., 2010). The contamination of *C. perfringens,* enterococci and *E. coli* in the environment may occur through sewage effluent discharges, manure and other animal waste run-off and the waste water from slaughterhouses (Balière et al., 2016; Jang et al., 2017; Mueller-Spitz et al., 2010). However, a few studies have reported the presence of *E. coli* in sand beaches and sediment (Alm et al., 2003; Fujioka et al., 1998) and total and faecal coliform bacteria have been found in pristine streams and in groundwater samples (Hazen, 1988). The content of enterococci per weight in mammal faeces, namely, humans and dogs, and in bird faeces, are usually between 10^4 and 10^6 and 10^4 and 10^8 bacteria and between 10^2 and 10^6 bacteria respectively (Boehm & Sassoubre, 2014).

Seasonal variations in bacterial quality have been reported in a few published studies. For example, elevated levels of coliforms and protozoans have been observed at weekends and on public holidays compared to weekdays, due to the larger number of people present in households and their subsequent waste discharges

(Jali & Ithoi, 2009). Similarly, preliminary observations in urbanized wetland inlets indicate a highly variable content of *E. coli* and coliforms, with higher levels of *E. coli* during dry periods and higher concentrations of total coliforms and COD during wet periods (Sharip et al., 2017). In this study, significant seasonal differences in bacterial quality were observed for *C. perfringens*. Seasonal trends for *E. coli*, faecal coliforms and total coliforms varied according to the lake, indicating that recent inputs of human faeces could be related to localize effects in the catchment area, such as those associated with on-site sewage facilities and pollution run-off. Sewage overflow or backflow from independent septic tanks that drain into urban storm water has been linked to *E. coli* detection in other lakes, such as Lake Michigan in the US and Putrajaya Lake in Malaysia (Bower et al., 2005; Sharip et al., 2016). Failing sewage pipes, resulting from cracks caused by invasive tree roots and shifting soils, have been reported as the main cause of *E. coli* leaks into the storm water run-off of the Dickinson Bayou watershed (Morrison et al., 2017). Leaky sewage lines, the illegal renovation of toilets or the inadequate construction of sewage facilities/ septic tanks, such as those at construction sites and in old housing areas, are known issues in developing countries that could contribute to the discharge or run-off of water contaminated with human faeces into storm water drains and waterbodies.

In terms of urbanization impact, variation in the bacterial parameters of the surrounding developments was only significant for *C. perfringens*. The lowest *C. perfringens* values were found in lakes located close to low levels of housing development. The values of *E. coli* and coliforms were fairly low in low-impact development areas. The presence of higher numbers of bacterial pathogens in highly populated areas suggests a larger contribution of human faeces, and possibly those of small domestic mammals and birds, to the bacterial contamination found in this study. This was dependent on the waterbody. Lakes found within highly populated areas are generally poor in water quality and have higher FIB content, due to human-related waste. For example, both L4 and L7 received discharges from nearby sewage treatment ponds that flowed into rivers and drains respectively before entering the ponds, while L14 received storm water drain discharges from ongoing construction sites nearby that intermittently caused pollution via an inadequate sewage treatment system. However, the faecal indicator bacteria of birds and mammals may affect some lakes. For example, L7 and L9 have an abundance of birds and otters that have become important elements in the urban habitat and in microbial contamination.

In this study, temperature, DO and TP explain the largest variation in the bacterial parameters. Elevated levels of *E. coli* are negatively correlated with temperature and this is consistent with the published findings (Sampson et al., 2006). *Escherichia coli* bacteria have been reported to survive for a longer period in cooler water (Sampson et al., 2006). This result is contrary to the

findings of other studies on the temperature dependence of the survival of *E. coli* (Staley et al., 2014). Surface water temperature is known to be a function of solar radiation: high solar irradiation, together with low cloud cover, increases the water column temperature and decreases bacterial density (Whitman et al., 2004). Despite the fact that the intestines of mammals have the optimal temperature for *E. coli*, around 36ºC-40ºC, the death rate for the bacteria is higher in warm temperatures (Jang et al., 2017). Dissolved oxygen (DO) and pH were both positively correlated with temperature. Higher temperatures can promote photosynthetic activities by algae and the release of DO. Dissolved oxygen (DO) also contributed to 20% of the variance in the bacterial parameters. High FIB values lead to the increased consumption of DO in water and a reduction in DO levels (Sadowsky & Whitman, 2011**)**. Some studies have shown that the best pH levels for *E. coli* are between 6 and 8, though elevated levels of *E. coli* were observed at lower pH values. High pH values or an acidic environment reduce the survival of *E. coli* (Sampson et al., 2006).

Clostridium perfringens, faecal coliforms and total coliforms were positively correlated with suspended solids and turbidity, indicating a linear relationship with the suspended sediments. The linear relationship suggests turbidity could be a proxy for water quality and a possible warning of pollution. FIB have a tendency to adsorb on suspended sediments or particles – the higher the particulate content the greater the increase in bacterial content. Additionally, turbid water reduces solar radiation and increases the survival of bacteria; reducing the bacteria die-off rate leads to a rise in FIB levels. The distribution of faecal pollution, namely, *C. perfringens* spores, was found to be associated with suspended sediments in the near-shore waters of Lake Michigan (Mueller-Spitz et al., 2010).

The NLWQS recommended levels of *E. coli* and enterococci are 100 cfu100 ml^{-1} and 33 cfu100 ml^{-1}, respectively. The level for *E. coli* was based on the values set in the Putrajaya Lake Water Quality Standard (Majizat et al., 2016), which provided a good indicator for monitoring fresh faecal contamination in the lake due to the short survival time. The criteria for enterococci were based on United States Environmental Protection Agency criteria from established work on the relationship between enterococci density and the gastrointestinal illness rate among bathers on freshwater beaches in the US (United States Environmental Protection Agency [USEPA], 2012). The use of both *E. coli* and enterococci in the water standards ensures the protection of human health through the detection and the control of FIB. Additionally, *C. perfringens* spores are some of the best available parameters for indicating potential remote pollution due to their ability to resist environmental stress. In this study, *C. perfringens* concentrations were observed in all lakes and ranged from 5 to 330 cfu100 ml^{-1}. The highest values of *C. perfringens* were found in L6, which is the furthest

downstream of the Klang river basin lakes, indicating possible long-term depositions. Byamukama et al. (2005) showed that the combination of *E. coli* and *C. perfringens* spores in current use formed a good basis for detecting both recent and remote faecal pollution events in environments with low physical turnover, such as lakes and ponds. Our work has shown variation in the *E. coli* and *C. perfringens* concentrations in different lakes, with significant correlation of the two species. Both bacteria were strongly associated with pathogenic and faecal contamination (Rodrigues & Cunha, 2017) and selecting both parameters can provide a useful indicator of faecal pollution in waterbodies (Byamukama et al., 2005). Monitoring both *E. coli* and *C. perfringens,* will enable a better understanding of the short-term and long-term impacts of faecal contamination in waterbodies and microbial persistence in the environment (Abia et al., 2015). This study found that faecal coliforms were highly correlated with *E. coli* and that total coliforms (supported by the rationale of the NLWQS recommendation of faecal coliforms as an optional monitoring parameter), were more useful for purposes of categorization (Sharip & Suratman, 2017). The use of *E. coli*, *C. perfringens* and enterococci was intended to increase public protection by ensuring that other biological indicators could be tested should one parameter fail to be detected. These FIBs can also be used as surrogate indicators of other pathogenic microorganisms, such as *Cryptosporidium* and *Giardia* (Brookes et al., 2005). The use of multiple bacterial indicators will increase reliability when assessing the risk of microbial contamination in waterbodies used for recreation and drinking water.

CONCLUSIONS

Overall, all urban ponds and lakes had significant microbial contamination, possibly of human origin. Among the microbial parameters, only *C. perfringens* significantly differed between seasons and according to urbanization impact. *Escherichia coli* values were significantly higher in a few waterbodies during the wet season. No correlation was observed between microbial quality and surface area or shoreline length. Temperature, dissolved oxygen (DO), total phosphorus (TP) and transparency are some of the parameters that are associated with the bacterial parameters. Further work is needed to control faecal pollution in ponds and to improve lake water quality before the lakes can be used for direct body-contact recreational purposes. In addition, the proposal to extract water stored in these waterbodies for use as an alternative water supply must consider the spatial and temporal variation in the bacterial content and the water quality of the waterbodies, to ensure cost-effective treatment technologies.

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Associations between the Presence of Bacteria and the Physico-Chemical Parameters of Water in Peat Swamp Forest, Paddy Field and Oil Palm Plantation in North Selangor, Malaysia

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ABSTRACT

This study determines the associations between the presence of bacteria and water physico-chemical parameters in peat swamp forest, paddy field and oil palm plantation in north Selangor, Malaysia. Sampling of bacteria and water physico-chemical parameters were conducted from four sites in the peat swamp forest, two sites each in paddy field and oil palm plantations. Oil palm plantation recorded the highest bacterial diversity (Shannon's H = 3.3713) and richness ($I_{Margalef}$ = 11.5955), while peat swamp forest showed highest bacterial evenness (Pielou's e = 0.9526). A total of 3,421 bacterial isolates from 39 bacterial species were obtained, which comprised of 11 Gram-positive and 28 Gram-negative bacteria. The highest number of bacteria was recorded in the oil palm plantation (1,552 isolates from 38 species), followed by the paddy field (1,191 isolates from 30 species) and the peat swamp forest (678 isolates from 22 species). In general, the most abundant bacteria was *Escherichia coli* (333 isolates; 9.73 %), followed by *Salmonella* spp. (288 isolates; 8.42 %), and *Streptococcus agalactiae* (252 isolates; 7.37 %). Moreover, *E. coli* recorded the highest isolated bacterium in the peat swamp forest (10.47%), paddy field (10.66%) and the oil palm plantation (8.7%). Inconsistent association was observed between the water physico-chemical parameters and the presence of bacteria in all studied habitats. However, multivariate analyses showed that water temperature, NH_3-N, Cl_2, DO, EC, SO_4 and PO_4 were able to influence

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the bacterial presence. This study showed that oil palm plantation and paddy field had the highest number of isolates, species, and bacterial concentrations due to the extensive anthropogenic activities in these areas.

Keywords: Association, bacteria, north Selangor peat swamp forest, water physico-chemical parameters

INTRODUCTION

The north Selangor peat swamp forest (NSPSF) is located on a flat coastal plain in the northern part of Selangor, Malaysia. The peat soil of NSPSF has high content of organic materials in various stages of decomposition, highly acidic with pH as low as 3.5, and contains limited amounts of nutrients but high carbon content (Global Environment Centre [GEC], 2014; Sule et al., 2016; Yule & Gomez, 2008). Currently, the NSPSF is the most extensively cleared peat swamp forest in Peninsular Malaysia. This is partly due to the proximity to the Integrated Agricultural Development Project, a paddy production scheme, which is the single biggest agricultural investment in the state. The NSPSF also suffers large-scale conversion to oil palm plantations, which is presently the biggest threat to peat swamp forests in the Southeast Asian region (Koh et al., 2011).

Numerous studies revealed a diverse microbial flora in Malaysian peat swamps (Jackson et al., 2009; Yule & Gomez, 2008). Jackson et al. (2009) employed molecular techniques to study the microbial communities of the peat sediment in NSPSF and found that the microbial communities are dominated by Acidobacteria and Crenarchaeota, with Archaea is limited to, but dominating the deeper samples. Moreover, they also revealed the lack of methanogenic bacteria in the microbial communities of NSPSF. This was supported by stable C isotope analyses of the peat which revealed depleted values of ^{13}C (Yule & Gomez, 2008). Fish from the same peat swamp also recorded lower ^{13}C values than those from freshwaters, indicating that the bacteria that respired carbon were being assimilated throughout the aquatic food web (Yule & Gomez, 2008). Moreover, gut analyses showed the most abundant invertebrates (mayfly and chironomid larvae) mainly ingested fine particulate organic matters that were largely composed of bacteria. These in turn were eaten by carnivorous invertebrates and fishes (Yule & Gomez, 2008). The importance of these is that the bacteria form the base of the peat swamp food web, illustrating a relationship between fish and bacteria in water and sediment.

However, previous studies only focussed on the dominant peat sediment bacterial communities and the leaf-degrading bacteria using molecular techniques and C isotope analyses. Studies on the diversity and distribution of bacterial species in sediment, water and fishes in peat swamp that are usually used as bio-indicators are generally lacking. Thus, this study determines the bacterial abundance and diversity in peat swamp forest, paddy field and oil palm plantation in north Selangor, while at the same time identifies the impact of water physico-chemical parameters on their presence.

MATERIALS AND METHODS

Study Area

NSPSF consisted of four forest reserves, namely the Raja Musa, Sungai Karang, Bukit Belata Extension and Sungai Dusun/ Wildlife Reserves. There was a stretch of paddy fields in NSPSF, which is one of the primary rice granary area in Malaysia, covering an area of 18980 hectares. An extensive area of the peat swamp forest has been converted to oil palm plantation, but remained a part of the NSPSF (GEC, 2014; Sule, 2016; Sule et al., 2016). The flora of NSPSF consisted of very tolerant tree species with relatively low diversity. The main sources of water entering the NSPSF were the rainfall and occasional water overflow from the Bernam River, Kuala Selangor. The NSPSF has a mean annual rainfall ranging from 1359 to 2480 mm, a mean temperature of 27°C and a mean relative humidity of 79.3%. The rainfall varies with distinct seasons of the year, wet/ rainy seasons (March-April and October-November) and relatively dry seasons (January-February and May-September) (GEC, 2014).

Eight sampling sites, comprising four sites in the peat swamp, and two each in the paddy field and oil palm plantation were selected. The sites were located within the Kampung Sungai Sireh area, Tanjong Karang, such that all peat swamp sites were on one side separated from the paddy field and oil palm plantation sites by the Tengi River, Tanjong Karang, along its entire length (Figure 1). Samplings were done thrice for water physicho-chemical analyses, fish collection and bacterial examination during the dry month (June 2015), relatively high rainfall month (October 2015) and a moderately dry month (January, 2016). A total of 24 sampling points, three points at each sampling site, were selected for this study.

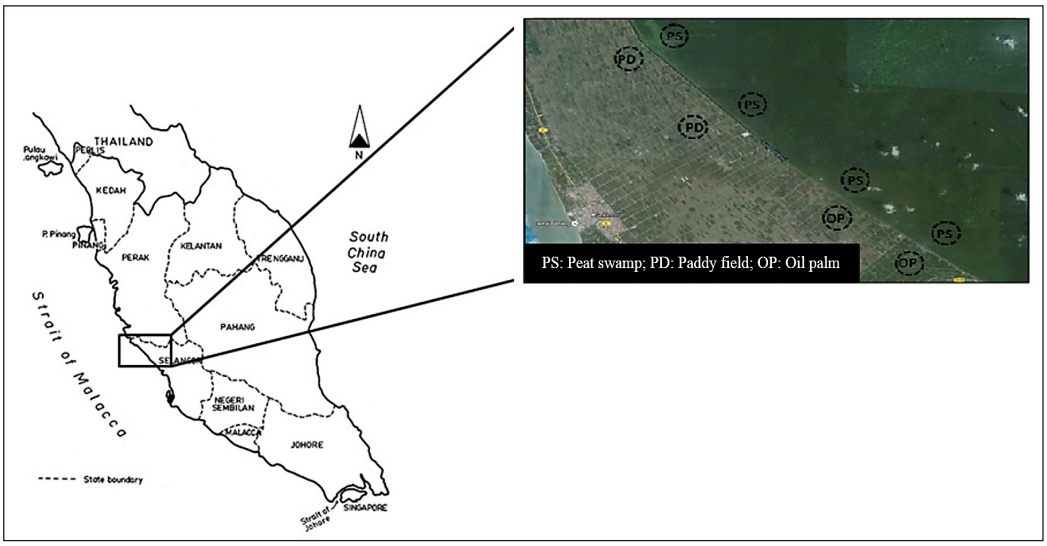

Figure 1. Map of Peninsular Malaysia with enlarged view of north Selangor peat swamp forest and study sites

Peat Swamp Forest

Four sampling sites in peat swamp forest were separated at ~ 200 m. Site 1 was located at 3° 34' 40.5444'' N, 101° 7' 0.4152'' E; site 2 at 3° 33' 7.1712'' N, 101° 8' 36.15'' E; site 3 at 3° 31' 9.4188'' N, 101° 10' 43.7736'' E; and site 4 at 3° 29' 45.7692'' N, 101° 12' 37.8864'' E. Site 1 had undergone significant logging, and dominated by shrubs with only very few trees present. During the third visit to the site, we noticed further degradation, where majority had been cleared and burnt. A major dumpsite was within the proximity of the site. Sites 2 and 3 had also undergone some logging, but had more vegetation cover than site 1. Site 4 appeared to be pristine, preserved in its natural form and untouched. The site was almost completely covered with vegetation, with many mature dipterocarp and other trees. It was relatively hidden and difficult to access.

Paddy Field

Two sampling sites in paddy field were also separated by ~ 200 m, and were ~ 500 m away from the peat swamp forest sites. Site 1 was located at 3° 34' 15.4164'' N, 101° 6' 43.2864'' E and site 2 at 3° 32' 40.8012'' N, 101° 8' 23.892'' E. These sites were typically artificial freshwater swamps dominated by the swamp grass with shrubs at the bank. The entire site areas were completely exposed to direct sunlight without vegetation cover. These sites were converted from the peat swamp forest as evident in the peat characteristics retained in the soil and water. The sites were characterised by the presence of trenches and screens firmly or loosely placed at interval. There were networks of large irrigation canals leading from the paddy sites to the Tengi River. Within the proximity of each site were human dwellings and other agricultural infrastructures. Intense application of insecticides/pesticides was observed during visits to the sites.

Oil Palm Plantation

Two sites within the oil palm plantation were also separated by ~ 200 m, and were ~ 500 m away from the peat swamp forest site. Site 1 was located at 3° 30' 41.7384'' N, 101° 10' 32.2068'' E, and ~ 300 m away from paddy site 2. Meanwhile, site 2 was located at 3° 29' 19.8816'' N, 101° 12' 31.5864'' E. The sites were dominated by the oil palm plants. The sites were flooded with water during each visit but there were irrigation canals leading to the Tengi River at each site. Site 1 was close to human settlement. The water was visibly polluted with domestic effluents and plastics. The irrigation canal in the oil palm plantation site 1 received direct discharge of waste water. Site 2 was relatively further away from human settlement. Although no direct effluent discharge into the canals was observed, the water was visibly polluted. Site 2 was used as a major litter site.

Water and Sediment Samples for Bacteriological Analyses

Prior to the measurement of water physico-chemical parameters, the water and sediment samples for bacteriological analyses were collected in triplicate from each site, totaling

24 samples for each sampling season. A total of 200 mL of the water sample was collected aseptically from 15 cm below the water surface using sterile polyethylene bottles, and immediately placed in an icebox for transportation to the laboratory. About 100 g of the sediment sample was collected from each station using a sterile scoop, transferred into sterile plastic bag, then immediately placed in an icebox and transported to the laboratory for further processing.

Fish Samples for Bacteriological Analyses

Twenty-four fish samples, or three fish from each site were collected using the scoop nets. Collected fish were transferred into a mini aquarium containing water from the site of collection, and immediately transferred to the laboratory for bacteriological analysis. In this study, we used three spot gourami *Trichopodus trichopterus*, as the target fish due to its availability in all of the three sampling areas in this study.

Bacterial Isolation from Water and Sediment

A total of 1 mL of the water sample and 1 g of the sediment sample was serially diluted to 10^{-6} using sterilized deionized water under complete aseptic condition. Briefly, six dilution tubes and petri dishes containing tryptic soy agar (TSA) (Merck, Darmstadt, Germany) were labelled from 1 to 6 accordingly. Nine mL of sterilized deionized water was transferred into each tube with the aid of a sterile pipette. The original water sample was vortexed. Then, 1 mL of the water sample was transferred into tube 1 and the contents were mixed vigorously.

A total of 1 g of the sediment sample was measured by transferring into pre-weighed sterilized dilution tube 1 containing 9 mL of sterilized deionized water and carefully weighed. The content was mixed vigorously. One mL of the solutions was then transferred from tube 1 to tube 2 using a sterile pipette and the contents were mixed vigorously. The same process was repeated for tubes 3, 4, 5 and 6 to give dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} respectively. Plates of TSA were then inoculated with 1 mL of the dilution from water and sediment in triplicate and incubated for 24 - 48 h at 37°C. Colony counts were performed and the results were expressed as CFU/mL and CFU/g of water and sediment respectively.

Bacterial Isolation from Fish

The skin, gill and intestine of the fish were used for bacterial analysis. The collected fish were euthanized with 250 mg/L of Tricane Methanesulfonate (MS 222), according to methods approved by the Institutional Animal Care and Utilization Committee, Universiti Putra Malaysia. A swab was taken from a 1 cm^2 surface area at the right abdomen of the fish. The swab was diluted in 10 mL of sterilized deionized water. The fish surfaces were then swabbed with 90% ethyl alcohol for surface sterilization prior to gill and intestine samplings. The gill was completely removed and macerated in a sterilized ceramic mortar. One gram of the sample was then measured by transferring

into a pre-weighed sterilized dilution tube containing 9 mL of sterilized deionized water and carefully weighed, and the content was mixed vigorously. The intestine was also removed completely, macerated in sterilized mortar, and 1 g was weighted by transferring into a pre-weighed sterilized dilution tube containing 9 mL of sterilized deionized water and carefully weighed, and the content mixed vigorously. All samples were then serially diluted to 10^{-6} using sterilized deionized water as previously described (Al-Harbi, 2003; Al-Harbi & Uddin, 2003). One mL of the dilution was then inoculated on plates of TSA in triplicate and incubated for 24 - 48 h at 37°C. Colony counts were performed and the results were expressed as CFU/cm^2 for skin, and CFU/g for gill and intestine.

Bacterial Identification from Water, Sediment and Fish

All bacterial growth isolates were sub-cultured on nutrient agar (NA) (Merck) for 24 – 48 h at 37°C to obtain pure isolates. All pure isolates were identified for their Gram staining, following characterization of colony size, structure, shape, elevation, edge, surface, opacity and colour. Thereafter, presumptive biochemical identification tests of oxidase, catalase, motility, amylase, gelatinase, lipase, indole, H_2S production, and nitrite reduction were performed.

Identification of isolates to genus or species level was done using Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). In addition, Gram-positive cocci and catalase-negative isolates were identified to species level using API® 20 Strep (bioMérieux, Marcy l'Etoile, France), Gram-positive cocci and catalase-positive isolates were identified using API® 20 Staph (bioMérieux), and Gram-negative rod were identified using API® 20E (bioMérieux).

Following bacterial identification from water, sediment and fish, the bacterial Shannon-Weaver diversity index (*H'*) (Shannon & Weaver, 1963), Pielou's evenness index (*e*) (Pielou, 1969) and Margalef's richness index ($I_{Margalef}$) (Margalef, 1958) were calculated. The indexes readings were pooled from the water, sediment and fish results. For better understanding and comparison, the results were also combined from all three sampling times and presented as mean for each habitat studied.

Water Physico-Chemical Analyses

The dissolved oxygen (DO), electrical conductivity (EC), pH, salinity, temperature, and total dissolved solids (TDS) were measured *in situ* using an YSI 556 MPS probe (YSI Incorporation, NY, USA). A total of 500 mL of water sample was collected in sterilized polyethylene sampling bottle in replicate from 15 cm below the water surface at each sampling site and transferred into an icebox. The samples were immediately transported to the laboratory. The nitrite (NO_3-N), ammonia-nitrogen (NH_3-N), chlorine (Cl_2), sulphate (SO_4), and phosphate (PO_4) concentration were measured using a DR900 Multiparameter Handheld Calorimeter (Hach Company, Loveland, Colorado, USA).

Statistical Analyses

The data for water physico-chemical parameters were tested for fitness to a normal distribution by the Shapiro-Wilk test, followed by ANOVA and Tukey's pairwise comparisons to test for significant difference of the water physico-chemical data between the three habitats (IBM SPSS, Version 22.0). Statistical significant difference was determined at $P< 0.05$.

In order to identify the relationships between water physico-chemical parameters and bacterial composition, Principal Component Analysis (PCA) was performed using IBM SPSS to reduce variable numbers in the dataset by combining highly correlated variables into factors, while retaining the variability in the data. This was based on the principle that dataset comprised of numerous variables was likely to be redundant if two or more variables were highly correlated with each other. PCA extraction was based on eigenvalues of 1 or greater, which was considered significant. Factor loadings of > 0.75, 0.75–0.50, and 0.50–0.00 were classified as strong, moderate, and weak respectively. Data reduction was performed on the eleven measured water physico-chemical parameters.

Then, water physico-chemical parameters variables that showed variation within the study area were utilized in determining the relationships between water physico-chemical parameters and bacterial composition. The relationships were identified using Canonical Component Analysis (CCA). The CCA was performed using XLSAT add-in for Microsoft Excel (Office 365, Version 2016). The significance of each variable was tested using CCA XLSTAT-ADA with 5000 permutations at a significance level of 5%. Results were presented using canonical biplots and other descriptive statistics.

RESULTS

Bacterial Counts from Water, Sediment and Fish

There was a significant difference ($P<0.05$) between the bacterial concentration in sediments of peat swamp forest, paddy field and oil palm plantation (Table 1). Meanwhile, peat swamp forest had significantly ($P<0.05$) lower bacterial counts in water, fish body surface, gill and intestine. However, no significant difference ($P>0.05$) existed between bacterial counts in water, fish body surface, gill and intestine

Table 1
Bacterial concentration in sediment, water, gill, body surface and intestine of fish

Habitat	Sediment ($\times 10^8$ cfu g^{-1})	Water ($\times 10^8$ cfu mL^{-1})	Fish body surface ($\times 10^8$ cfu cm^{-2})	Fish gill ($\times 10^8$ cfu g^{-1})	Fish intestine ($\times 10^8$ cfu g^{-1})
Peat swamp	0.09 ± 0.02^{aA}	0.59 ± 0.39^{aB}	0.71 ± 0.24^{aB}	1.28 ± 0.36^{aC}	3.87 ± 1.12^{aD}
Paddy field	0.30 ± 0.11^{bA}	1.15 ± 0.40^{bB}	1.16 ± 0.27^{bB}	2.19 ± 0.27^{bC}	5.12 ± 0.93^{bD}
Oil palm	0.48 ± 0.13^{cA}	1.71 ± 0.65^{bB}	1.53 ± 0.54^{bB}	2.35 ± 0.40^{bC}	6.33 ± 1.44^{bD}

Values with different superscript of lower case letters of the same columns are significantly different at $P<0.05$
Values with different superscript of capital letters of the same rows are significantly different at $P<0.05$

between paddy field and oil palm plantation. Moreover, the bacterial counts in sediments of all habitats were significantly ($P<0.05$) lower compared to bacteria counts in water. No significant difference ($P>0.05$) existed between the bacterial counts in water and fish body surface, but significant difference ($P<0.05$) was observed between the bacterial counts in the fish body surface, gill and intestine for all habitats.

Taxonomic Composition of Isolated Bacteria

Bacterial community structure analyses showed that oil palm plantation recorded the highest bacterial diversity (Shannon's $H = 3.3713$) and richness ($I_{Margalef} = 11.5955$), while peat swamp forest showed the highest bacterial evenness (Pielou's $e = 0.9526$) (Table 2).

In general, 39 species of bacteria were isolated throughout the study comprising of 11 Gram-positive and 28 Gram-negative bacteria. The highest number of bacteria was recorded for the oil palm plantation (1,552 isolates from 38 species), followed by the paddy field (1,191 isolates from 30 species), and the peat swamp forest (678 isolates from 22 species). The most abundant bacterial species was *Escherichia coli* (333 isolates; 9.73 %), followed by *Salmonella* spp. (288 isolates; 8.42 %) and *Streptococcus agalactiae* (252 isolates; 7.37 %). Meanwhile the least isolated bacterial species were *Aerococcus urinae* (9 isolates; 0.26 %), *Aeromonas veronii* (9 isolates; 0.26 %), and *Yersinia pseudotuberculosis* (9 isolates; 0.26 %).

The highest number of isolates were recorded in the fish intestine from the peat swamp forest (212 isolates), the paddy field (374 isolates) and the oil palm plantation (484 isolates), while the least number of isolates were recorded in the sediment from peat swamp forest (54 isolates), the paddy field (96 isolates) and the oil palm plantation (118 isolates).

In peat swamp forest, the most isolated bacteria from the sediment and fish intestine were *Bacillus* spp. (13 isolates; 24.07%) and *Salmonella* spp. (21 isolates; 9.91%). However, *E. coli* dominated the isolation of bacteria from water (19 isolates; 14.96%), the fish body surface (17 isolates; 12.78%) and the fish gill (17 isolates; 11.18%). In the paddy field area, *E. coli* was also dominated in the water (32 isolates; 12.50%) and fish gill (28 isolates; 11.11%). However, *Enterococcus pseudoarium*, *Salmonella* spp. and *S. agalactiae* were commonly isolated from the sediment (15 isolates; 15.63%), fish body surface (24 isolates; 11.27%) and fish intestine (37 isolates; 9.89%), respectively. However, for oil palm plantation area, *E. coli* was mostly isolated from the sediment (15 isolates; 12.71%), water (29 isolates; 9.45%), and the fish body surface (33 isolates; 10.61%). Similar isolation rate of *Salmonella* spp. and *S. agalactiae* were recorded in fish gill (31 isolates; 9.34%), while *S. agalactiae* was also dominantly isolated from the fish intestine (42 isolates; 8.68%). The list of isolated bacterial species, their number, percentages, and details of their sources from each habitat are presented in Tables 3 - 6.

Table 2
Bacterial community structure in peat swamp forest, paddy field and oil palm plantation expressed as diversity, evenness and richness index

Habitat	Community structure index		
	Shannon's *H*	Pielou's *e*	$I_{Margalef}$
Peat swamp	2.9445	0.9526	7.4173
Paddy field	3.1826	0.9357	9.4281
Oil palm	3.3713	0.9268	11.5955

Shannon's *H* = Shannon-Weiner diversity index; Pielou's *e* = Pielou's evenness index; $I_{Margalef}$ = Margalef's richness index

Table 3
List of bacteria, abbreviation used, number and percentage of isolates from peat swamp forest, paddy field and oil palm plantation

Bacteria	Abbrev.	Peat swamp (n = 678)		Paddy field (n = 1,191)		Oil palm (n = 1,552)		Total (N = 3,421)	
		No.	%	No.	%	No.	%	No.	%
Aerococcus urinae	A.uri	-	-	-	-	9	0.58	9	0.26
Aeromonas hydrophila	A.hyd	-	-	23	1.93	31	2.00	54	1.58
Aeromonas veronii	A.ver	-	-	-	-	9	0.58	9	0.26
Bacillus spp.†	Baci	59	8.70	78	6.55	92	5.93	229	6.69
Budvicia aquatica	B.aqu	37	5.46	51	4.28	47	3.03	135	3.95
Citobacter diversus	C.div	-	-	27	2.27	40	2.58	67	1.96
Citrobacter koseri	C.kos	32	4.72	57	4.79	65	4.19	154	4.50
Deinobacter grandis	D.gra	19	2.80	28	2.35	25	1.61	72	2.10
Deinococcus proteolyticus†	D.pro	9	1.33	17	1.43	15	0.97	41	1.20
Deinococcus radiopugnans†	D.rad	-	-	7	0.59	9	0.58	16	0.47
Edwardsiella tarda	E.tar	31	4.57	43	3.61	41	2.64	115	3.36
Enterobacter aerogenes	E.aer	-	-	9	0.76	15	0.97	24	0.70
Enterobacter cloacae	E.clo	11	1.62	31	2.60	37	2.38	79	2.31
Enterococcus cecorum†	E.cec	-	-	19	1.60	25	1.61	44	1.29
Enterococcus faecalis†	E.fae	-	-	13	1.09	19	1.22	32	0.94
Enterococcus pseudoarium†	E.pse	26	3.83	37	3.11	47	3.03	110	3.22
Escherichia coli	E.col	71	10.47	127	10.66	135	8.70	333	9.73
Escherichia coli 1	E.co1	-	-	34	2.85	47	3.03	81	2.37
Flavobacterium aquatile	F.aqu	19	2.80	29	2.43	48	3.09	96	2.81
Klebsiella oxytoca	K.oxy	28	4.13	37	3.11	46	2.96	111	3.24
Klebsiella pneumoniae subsp. *ozaenae*	K.pso	21	3.10	32	2.69	46	2.96	99	2.89
Klebsiella pneumoniae subsp. *pneumoniae*	K.psp	-	-	-	-	13	0.84	13	0.38
Lactococcus lactis subsp. *lactis*†	L.lsl	-	-	-	-	15	0.97	15	0.44

Table 3 *(continue)*

Bacteria	Abbrev.	Peat swamp (n = 678)		Paddy field (n = 1,191)		Oil palm (n = 1,552)		Total (N = 3,421)	
		No.	%	No.	%	No.	%	No.	%
Leuconostoc spp.†	Leuc	-	-	15	1.26	29	1.87	44	1.29
Pantoea spp.	Pant	19	2.80	24	2.02	32	2.06	75	2.19
Pragia fontium	P.fon	27	3.98	37	3.11	42	2.71	106	3.10
Proteus mirabilis	P.mir	16	2.36	23	1.93	31	2.00	70	2.05
Proteus vulgaris	P.vul	14	2.06	19	1.60	29	1.87	62	1.81
Rahnella aquatilis	R.aqu	29	4.28	39	3.27	43	2.77	111	3.24
Salmonella choleraesius subsp. *choleraesius*	S.csc	-	-	-	-	11	0.71	11	0.32
Salmonella enterica	S.ent	-	-	19	1.60	33	2.13	52	1.52
Salmonella spp.	Salm	63	9.29	103	8.65	122	7.86	288	8.42
Serratia fonticola	S.fon	23	3.39	47	3.95	52	3.35	122	3.57
Spirochaeta aurantia	S.aur	19	2.80	-	-	-	-	19	0.56
Staphylococcus aureus subsp. *aureus*†	S.asa	-	-	-	-	15	0.97	15	0.44
Staphylococcus spp.†	Stap	58	8.55	77	6.47	101	6.51	236	6.90
Streptococcus agalactiae†	S.aga	47	6.93	89	7.47	116	7.47	252	7.37
Vibrio cholerae	V.cho	-	-	-	-	11	0.71	11	0.32
Yersinia pseudotuberculosis	Y.pse	-	-	-	-	9	0.58	9	0.26

†: Indicate Gram-positive
-: Absent

Table 4
List of bacteria, abbreviation used, number and percentage of isolates from sediment, water, gill, body surface and intestine of fish in peat swamp forest

Bacteria	Abbrev.	Sediment (n = 54)		Water (n = 127)		Body surface (n = 133)		Gill (n = 152)		Intestine (n = 212)	
		No.	%	No.	%	No.	%	No.	%	No.	%
Bacillus spp.†	Baci	13	24.07	9	7.09	12	9.02	8	5.26	17	8.02
Budvicia aquatica	B.aqu	2	3.70	3	2.36	10	7.52	9	5.92	13	6.13
Citrobacter koseri	C.kos	0	0.00	3	2.36	7	5.26	9	5.92	13	6.13
Deinobacter grandis	D.gra	1	1.85	2	1.57	4	3.01	5	3.29	7	3.30
Deinococcus proteolyticus†	D.pro	0	0.00	2	1.57	3	2.26	2	1.32	2	0.94
Edwardsiela tarda	E.tar	3	5.56	7	5.51	6	4.51	4	2.63	11	5.19
Enterobacter cloacae	E.clo	0	0.00	3	2.36	1	0.75	2	1.32	5	2.36
Enterococcus pseudoarium†	E.pse	1	1.85	4	3.15	5	3.76	5	3.29	11	5.19
Escherichia coli	E.col	5	9.26	19	14.96	17	12.78	17	11.18	13	6.13
Flavobacterium aquatile	F.aqu	2	3.70	3	2.36	4	3.01	6	3.95	4	1.89
Klebsiella oxytoca	K.oxy	1	1.85	6	4.72	4	3.01	5	3.29	12	5.66

Table 4 *(continue)*

Bacteria	Abbrev.	Sediment (n = 54)		Water (n = 127)		Body surface (n = 133)		Gill (n = 152)		Intestine (n = 212)	
		No.	%	No.	%	No.	%	No.	%	No.	%
Klebsiella pneumoniae subsp. *ozaenae*	K.pso	0	0.00	3	2.36	3	2.26	8	5.26	7	3.30
Pantoea spp.	Pant	3	5.56	2	1.57	3	2.26	5	3.29	6	2.83
Pragia fontium	P.fon	5	9.26	12	9.45	5	3.76	2	1.32	3	1.42
Proteus mirabilis	P.mir	0	0.00	3	2.36	4	3.01	3	1.97	6	2.83
Proteus vulgaris	P.vul	2	3.70	2	1.57	2	1.50	4	2.63	4	1.89
Rahnella aquatilis	R.aqu	2	3.70	5	3.94	7	5.26	6	3.95	9	4.25
Salmonella spp.	Salm	4	7.41	13	10.24	11	8.27	14	9.21	21	9.91
Serratia fonticola	S.fon	0	0.00	5	3.94	4	3.01	5	3.29	9	4.25
Spirochaeta aurantia	S.aur	1	1.85	2	1.57	3	2.26	9	5.92	4	1.89
Staphylococcus spp.†	Stap	6	11.11	9	7.09	11	8.27	13	8.55	19	8.96
Streptococcus agalactiae†	S.aga	3	5.56	10	7.87	7	5.26	11	7.24	16	7.55

†: Indicate Gram-positive

Table 5
List of bacteria, abbreviation, number and percentage of isolates from sediment, water, gill, body surface and intestine in paddy field

Bacteria	Abbrev.	Sediment (n = 96)		Water (n = 256)		Body surface (n = 213)		Gill (n = 252)		Intestine (n = 374)	
		No.	%	No.	%	No.	%	No.	%	No.	%
Aeromonas hydrophila	A.hyd	10	10.42	4	1.56	3	1.41	5	1.98	10	2.67
Bacillus spp.†	Baci	4	4.17	15	5.86	13	6.10	18	7.14	22	5.88
Budvicia aquatica	B.aqu	4	4.17	11	4.30	7	3.29	13	5.16	16	4.28
Citobacter diversus	C.div	9	9.38	6	2.34	4	1.88	3	1.19	10	2.67
Citrobacter koseri	C.kos	3	3.13	11	4.30	7	3.29	9	3.57	21	5.61
Deinobacter grandis	D.gra	0	0	6	2.34	4	1.88	6	2.38	9	2.41
Deinococcus proteolyticus†	D.pro	0	0	5	1.95	3	1.41	2	0.79	7	1.87
Deinococcus radiopugnans†	D.rad	2	2.08	3	1.17	2	0.94	1	0.40	1	0.27
Edwardsiela tarda	E.tar	1	1.04	7	2.73	6	2.82	13	5.16	15	4.01
Enterobacter aerogenes	E.aer	1	1.04	2	0.78	1	0.47	1	0.40	4	1.07
Enterobacter cloacae	E.clo	1	1.04	4	1.56	5	2.35	9	3.57	12	3.21
Enterococcus cecorum†	E.cec	0	0	4	1.56	2	0.94	5	1.98	7	1.87
Enterococcus faecalis†	E.fae	1	1.04	3	1.17	1	0.47	4	1.59	5	1.34
Enterococcus pseudoarium†	E.pse	15	15.63	6	2.34	6	2.82	10	3.97	14	3.74
Escherichia coli	E.col	2	2.08	32	12.50	21	9.86	28	11.11	31	8.29
Escherichia coli 1	E.co1	2	2.08	12	4.69	5	2.35	7	2.78	8	2.14

Table 5 *(continue)*

Bacteria	Abbrev.	Sediment (n = 96)		Water (n = 256)		Body surface (n = 213)		Gill (n = 252)		Intestine (n = 374)	
		No.	%	No.	%	No.	%	No.	%	No.	%
Flavobacterium aquatile	F.aqu	3	3.13	6	2.34	5	2.35	11	4.37	5	1.34
Klebsiella oxytoca	K.oxy	0	0	8	3.13	7	3.29	10	3.97	9	2.41
Klebsiella pneumoniae subsp. *ozaenae*	K.pso	0	0	8	3.13	8	3.76	9	3.57	7	1.87
Leuconostoc spp.†	Leuc	2	2.08	6	2.34	2	0.94	2	0.79	5	1.34
Pantoea spp.	Pant	3	3.13	7	2.73	5	2.35	1	0.40	9	2.41
Pragia fontium	P.fon	2	2.08	8	3.13	9	4.23	7	2.78	10	2.67
Proteus mirabilis	P.mir	2	2.08	7	2.73	5	2.35	5	1.98	4	1.07
Proteus vulgaris	P.vul	4	4.17	5	1.95	5	2.35	3	1.19	4	1.07
Rahnella aquatilis	R.aqu	1	1.04	11	4.30	5	2.35	8	3.17	11	2.94
Salmonella enterica	S.ent	11	11.46	8	3.13	3	1.41	2	0.79	5	1.34
Salmonella spp.	Salm	3	3.13	13	5.08	24	11.27	21	8.33	34	9.09
Serratia fonticola	S.fon	6	6.25	14	5.47	11	5.16	7	2.78	12	3.21
Staphylococcus spp.†	Stap	3	3.13	11	4.30	15	7.04	15	5.95	30	8.02
Streptococcus agalactiae†	S.aga	10	10.42	13	5.08	19	8.92	17	6.75	37	9.89

†: Indicate Gram-positive

Table 6
List of bacteria, abbreviation, number and percentage of isolates from sediment, water, gill, body surface and intestine in oil palm plantation

Bacteria	Abbrev.	Sediment (n = 118)		Water (n = 307)		Surface (n = 311)		Gill (n = 332)		Intestine (n = 484)	
		No.	%	No.	%	No.	%	No.	%	No.	%
Aerococcus urinae	A.uri	0	0	6	1.95	1	0.32	0	0.00	2	0.41
Aeromonas hydrophila	A.hyd	1	0.85	8	2.61	4	1.29	7	2.11	11	2.27
Aeromonas veronii	A.ver	0	0	3	0.98	2	0.64	0	0.00	4	0.83
Bacillus spp.†	Baci	13	11.02	18	5.86	16	5.14	19	5.72	26	5.37
Budvicia aquatica	B.aqu	9	7.63	11	3.58	12	3.86	5	1.51	10	2.07
Citobacter diversus	C.div	3	2.54	6	1.95	10	3.22	7	2.11	14	2.89
Citrobacter koseri	C.kos	12	10.17	9	2.93	12	3.86	15	4.52	17	3.51
Deinobacter grandis	D.gra	1	0.85	3	0.98	5	1.61	6	1.81	10	2.07
Deinococcus proteolyticus†	D.pro	0	0.00	4	1.30	1	0.32	2	0.60	8	1.65
Deinococcus radiopugnans†	D.rad	0	0	2	0.65	1	0.32	1	0.30	5	1.03
Edwardsiella tarda	E.tar	1	0.85	7	2.28	9	2.89	8	2.41	16	3.31
Enterobacter aerogenes	E.aer	0	0	4	1.30	3	0.96	1	0.30	7	1.45
Enterobacter cloacae	E.clo	2	1.69	9	2.93	7	2.25	6	1.81	13	2.69

Table 6 *(continue)*

Bacteria	Abbrev.	Sediment (n = 118)		Water (n = 307)		Surface (n = 311)		Gill (n = 332)		Intestine (n = 484)	
		No.	%	No.	%	No.	%	No.	%	No.	%
Enterococcus cecorum†	E.cec	2	1.69	5	1.63	4	1.29	6	1.81	8	1.65
Enterococcus faecalis†	E.fae	0	0	3	0.98	1	0.32	4	1.20	11	2.27
Enterococcus pseudoarium†	E.pse	3	2.54	7	2.28	9	2.89	13	3.92	15	3.10
Escherichia coli	E.col	15	12.71	29	9.45	33	10.61	26	7.83	32	6.61
Escherichia coli 1	E.co1	0	0	13	4.23	9	2.89	14	4.22	11	2.27
Flavobacterium aquatile	F.aqu	5	4.24	9	2.93	12	3.86	11	3.31	11	2.27
Klebsiella oxytoca	K.oxy	2	1.69	8	2.61	12	3.86	7	2.11	17	3.51
Klebsiella pneumoniae subsp. *ozaenae*	K.pso	4	3.39	9	2.93	11	3.54	10	3.01	12	2.48
Klebsiella pneumoniae subsp. *pneumoniae*	K.psp	0	0.00	4	1.30	3	0.96	2	0.60	4	0.83
Lactococcus lactis subsp. *lactis*†	L.lsl	0	0	2	0.65	1	0.32	3	0.90	9	1.86
Leuconostoc spp.†	Leuc	1	0.85	8	2.61	6	1.93	5	1.51	9	1.86
Pantoea spp.	Pant	2	1.69	6	1.95	7	2.25	5	1.51	12	2.48
Pragia fontium	P.fon	4	3.39	8	2.61	7	2.25	10	3.01	13	2.69
Proteus mirabilis	P.mir	1	0.85	5	1.63	10	3.22	6	1.81	9	1.86
Proteus vulgaris	P.vul	1	0.85	6	1.95	5	1.61	7	2.11	10	2.07
Rahnella aquatilis	R.aqu	4	3.39	9	2.93	7	2.25	13	3.92	10	2.07
Salmonella choleraesius subsp. *choleraesius*	S.csc	0	0	5	1.63	2	0.64	1	0.30	3	0.62
Salmonella enterica	S.ent	0	0	5	1.63	7	2.25	10	3.01	11	2.27
Salmonella spp.	Salm	12	10.17	19	6.19	26	8.36	31	9.34	34	7.02
Serratia fonticola	S.fon	9	7.63	11	3.58	12	3.86	7	2.11	13	2.69
Staphylococcus aureus subsp. *aureus*†	S.asa	0	0	5	1.63	3	0.96	2	0.60	5	1.03
Staphylococcus spp.†	Stap	7	5.93	20	6.51	16	5.14	27	8.13	31	6.40
Streptococcus agalactiae†	S.aga	4	3.39	16	5.21	23	7.40	31	9.34	42	8.68
Vibrio cholerae	V.cho	0	0	3	0.98	1	0.32	2	0.60	5	1.03
Yersinia pseudotuberculosis	Y.pse	0	0	2	0.65	1	0.32	2	0.60	4	0.83

†: Indicate Gram-positive

Water Physico-Chemical Parameters

No significant difference ($P>0.05$) was observed for water temperature of the three habitats (Table 7). Peat swamp forest significantly ($P<0.05$) had the lowest water pH (3.68 ± 0.25) and DO (0.59 ± 0.17 mg L^{-1}), compared with paddy field and oil palm plantation. Higher mean of EC (204.85 ± 29.70 µS cm^{-1}) and TDS (0.13 ± 0.02 g L^{-1}) were recorded in the peat swamp forest,

but not significantly different (P>0.05) from the oil palm plantation. Peat swamp forest recorded highest salinity (0.05 ± 0.04 ppt) which was not significantly different (P>0.05) from the paddy field, but significantly (P<0.05) higher from oil palm plantation area. The highest NH_3-N (0.46 ± 0.04 mg L^{-1}) concentration was measured in the oil palm plantation area. The reading was significantly (P<0.05) higher compared to the peat swamp forest, but not for the paddy field. The lowest NO_3-N (0.002 ± 0.003 mg L^{-1}) was recorded in the peat swamp forest. However, the reading was not significantly different (P>0.05) from oil palm plantation, but significantly (P<0.05) lower compared to paddy field. Moreover, peat swamp forest recorded significantly (P<0.05) higher PO_4 (1.94 ± 0.20 mg L^{-1}), SO_4 (28.92 ± 20.40 mg L^{-1}) and Cl_2 (0.92 ± 0.36 mg L^{-1}) compared to the paddy field and oil palm plantation, respectively.

Table 7
Comparison of water quality parameters between the three different environments

	Peat swamp		Paddy field		Oil palm	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Temp (°C)	31.62 ± 2.70[a]	27.80 – 35.70	30.65 ± 2.23[a]	28.30 – 33.80	30.71 ± 1.80[a]	28.10 – 33.10
pH (1 – 14)	3.68 ± 0.25[a]	3.28 – 4.10	4.96 ± 0.46[b]	4.26 – 5.57	5.61 ± 0.62[b]	4.62 – 6.17
DO (mg L^{-1})	0.59 ± 0.17[a]	0.36 – 0.86	3.16 ± 0.56[b]	2.49 – 3.89	3.58 ± 0.30[b]	3.17 – 3.97
EC (µS cm^{-1})	204.85 ± 29.70[a]	155.70 – 271.00	92.94 ± 41.57[b]	61.00 – 156.03	108.12 ± 99.82[ab]	32.00 – 241.90
TDS (g L^{-1})	0.13 ± 0.02[a]	0.10 – 0.17	0.06 ± 0.03[b]	0.04 – 0.10	0.07 ± 0.07[ab]	0.02 – 0.18
Salinity (ppt)	0.05 ± 0.04[a]	0.01 – 0.12	0.02 ± 0.01[a]	0.01 – 0.03	0.01 ± 0.00[b]	0.01 – 0.12
NH_3-N (mg L^{-1})	0.35 ± 0.08[a]	0.23 – 0.45	0.45 ± 0.03[b]	0.41 – 0.50	0.46 ± 0.04[b]	0.39 – 0.50
NO_3-N (mg L^{-1})	0.002 ± 0.003[a]	0.000 – 0.008	0.005 ± 0.004[b]	0.000 – 0.009	0.003 ± 0.005[ab]	0.000 – 0.012
PO_4 (mg L^{-1})	1.94 ± 0.20[a]	1.56 – 2.30	0.37 ± 0.12[b]	0.22 – 0.56	0.38 ± 0.10[b]	0.28 – 0.56
SO_4 (mg L^{-1})	28.92 ± 20.40[a]	11.00 – 68.00	3.33 ± 1.21[b]	2.00 – 5.00	5.83 ± 3.19[b]	2.00 – 11.00
Cl_2 (mg L^{-1})	0.92 ± 0.36[a]	0.46 – 1.60	0.63 ± 0.72[b]	0.10 – 2.00	0.21 ± 0.10[b]	0.05 – 0.31

Comparison is between mean ± SD along the same row. Values with different superscript letters are significantly different at P <0.05

PCA and CCA of Water Physico-Chemical Parameters

For each habitat, PCA produced two axes that cumulatively explained the 88.25%, 97.70%, and 83.60% variations of water physico-chemical parameters in the habitats, respectively (Table 8). Out of the 11 water physico-chemical parameters evaluated, only four parameters were retained in each habitat. The water temperature, EC, NH_3-N, and Cl_2 were retained in peat swamp forest; the NH_3-N, PO_4, SO_4, and Cl_2 were retained

in paddy field; while the temperature, DO, EC, and SO_4 were retained in the oil palm plantation. Generally, factor loadings were classified as strong (> 0.75), moderate (0.75–0.50), or weak (0.50–0.00).

All four variables of water physico-chemical parameters of each habitat from the PCA were retained by CCA (Table 9). These variables were significant contributors to the variation in CCA's ordination. Four ordination axes were generated for the CCA in each habitat. The cumulative percentages for the first and second ordination axes were 44.26% and 71.52%, 39.68% and 76.58%, and 51.67% and 79.53%, for the peat swamp forest, paddy field, and the oil palm plantation, respectively. Only the first two axes are reported here, since these axes contributed the most to the ordination.

Table 8
Principal component loadings from principal component analysis of water quality parameters from peat swamp forest, paddy field and oil palm plantation

	Peat Swamp		Paddy Field		Oil Palm	
	C1	C2	C1	C2	C1	C2
Eigenvalue	2.501	1.029	2.763	1.145	1.754	1.590
Percentage variance explained	62.535	25.714	69.086	28.613	43.858	39.738
Cumulative variance explained	62.535	88.249	69.086	97.699	43.858	83.597
Temperature (°C)	0.875	0.195			-0.097	**0.840**
DO (mg L^{-1})					**0.873**	-0.324
EC (µS cm^{-1})	0.048	**0.994**			**0.911**	0.167
NH_3-N (mg L^{-1})	**0.949**	0.016	**0.971**	0.168		
PO_4 (mg L^{-1})			**0.977**	-0.110		
SO_4 (mg L^{-1})			-0.060	**0.996**	0.100	**0.911**
Cl_2 (mg L^{-1})	**0.913**	-0.052	**0.929**	-0.336		

Strong loadings > 0.70 in **bold**

Associations between Bacterial Presence and Water Physico-Chemical Parameters

The CCA ordination diagram showing the relationship between bacterial presence and water physico-chemical parameters in the peat swamp forest is presented in Figure 2. As revealed by the length of the vector, Cl_2 was the most important parameter influencing bacterial presence. This was followed by EC, NH_3-N and temperature. Bacterial species such as *Klebsiella oxytoca*, *Pantoea* spp., *Staphylococcus* spp., *Pragia fontium*, *Proteus vulgaris*, *Salmonella* spp., and *Klebsiella pneumoniae* showed positive correlation with the water parameters. In the same vein, species such as *E. coli*, *Flavobacterium aquatile* and *Proteus mirabilis* showed positive correlation with Cl_2. On the other hand, the species such as *Deinococcus preoteolyticus*, *Edwardsiella tarda*, *Enterococcus pseudoarium*, *Rahnella aquatilis* were negatively correlated with all the water physico-chemical parameters.

Table 9
Canonical correspondence analysis summary statistics of physico-chemical parameters of water

	Peat swamp				Paddy field				Oil palm			
	F1	F2	F3	F4	F1	F2	F3	F4	F1	F2	F3	F4
Eigenvalue	0.062	0.038	0.027	0.012	0.107	0.100	0.047	0.016	0.051	0.027	0.015	0.005
Constrained inertia (%)	44.261	27.260	19.578	8.901	39.677	36.904	17.477	5.942	51.673	27.856	14.962	5.509
Cumulative %	44.261	71.521	91.099	100.000	39.677	76.581	94.058	100.000	51.673	79.529	94.491	100.000
Regression coefficients:												
Temperature (°C)	1.209	1.107	0.771	0.110					0.039	0.039	1.088	0.313
DO (mg L^{-1})									0.723	0.266	1.228	-0.726
EC (µS cm^{-1})	0.421	-0.709	-0.169	-0.635					0.427	0.417	-0.647	0.948
NH_3-N (mg L^{-1})	-0.115	0.440	-2.485	-0.184	-1.828	0.241	0.066	3.335				
PO_4 (mg L^{-1})					-2.561	1.181	0.549	-2.799				
SO_4 (mg L^{-1})					1.140	-1.566	-0.209	-1.052	-0.008	0.379	-0.404	-1.004
Cl_2 (mg L^{-1})	-0.528	-1.563	1.521	0.997	4.795	-2.309	0.292	-0.837				

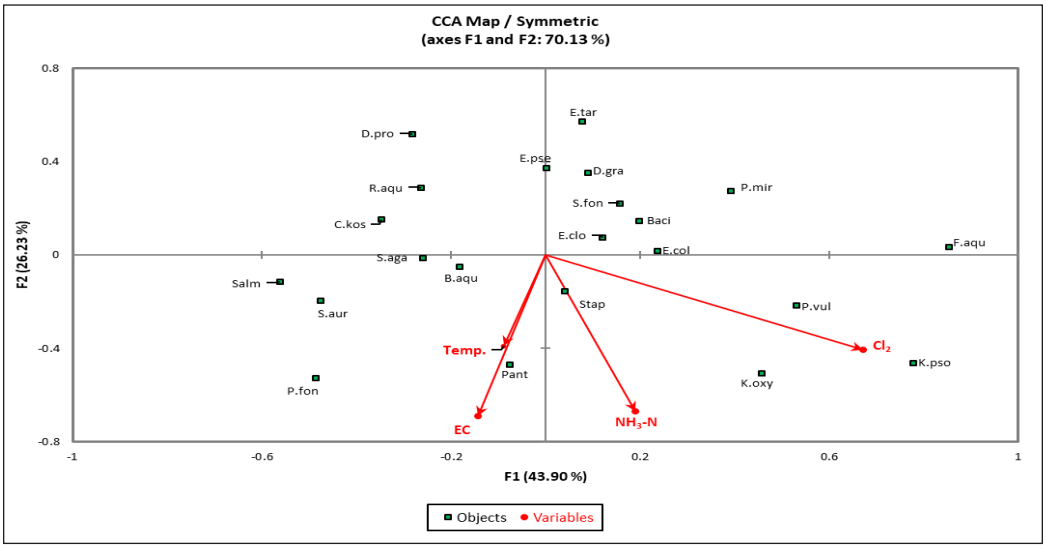

Figure 2. Canonical correspondence analysis ordination diagram showing the effect of water physico-chemical parameters on bacteria presence in peat swamp forest

Figure 3 presents the effects of water physico-chemical parameters on the bacterial presence in the paddy field. SO_4 is the most important parameter influencing the composition. Meanwhile, NH_3-N, PO_4 and Cl_2 displayed the same level of importance based on the vector lengths. Briefly, SO_4 was positively correlated with the species

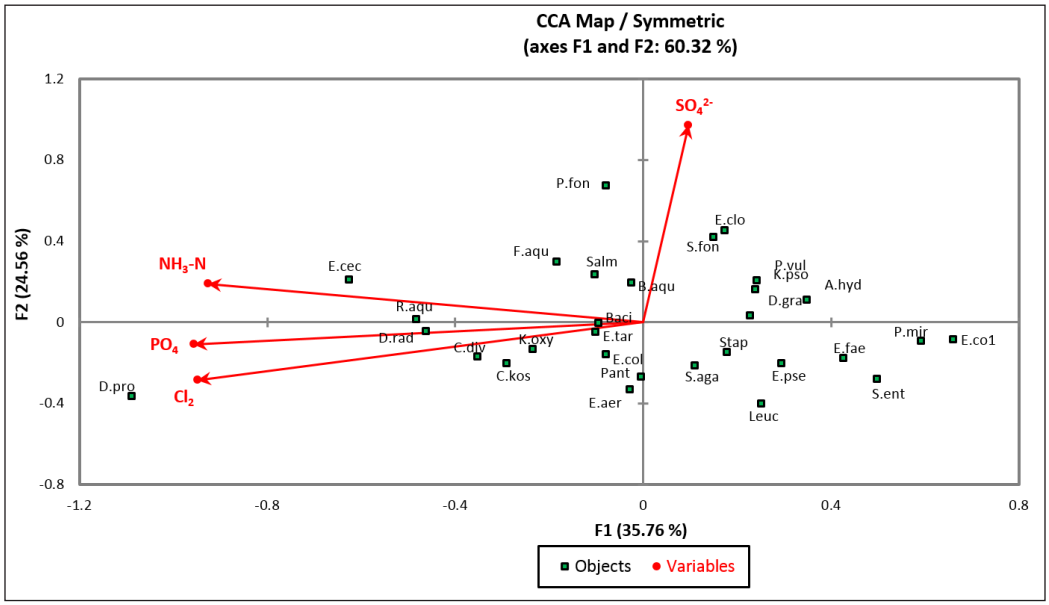

Figure 3. Canonical correspondence analysis ordination diagram showing the effect of water physico-chemical parameters on bacteria presence in paddy field

such as *P. fontium*, *F. aquatile, Serratia fonticola*, *Enterobacter cloacae*, *P. vulgaris*, and *Aeromonas hydrophila*, but negatively correlated with the bacterial species such as *E. coli*, *Leuconostoc* spp., *Streptococcus agalactiae*, *Staphylococcus* spp., and *Enterococcus faecalis*. However, NH_3-N, PO_4 and Cl_2 were positively correlated with *R. aquatilis*, *Deinococcus radiopugnans*, *D. proteolyticus*, *Enterococcus cecorum*, *Citrobacter diversus*, *Citrobacter koseri* and *K. oxytoca*, but negatively correlated with *S. agalactiae*, *Staphylococcus* spp., *P. vulgaris*, *P. mirabilis* and *Leuconostoc* spp.

In oil palm plantation, DO was the most important factor influencing bacterial composition, followed by SO_4, temperature and EC (Figure 4). DO and EC were positively correlated with *E. aerogenes*, *Budvicia aquatica*, *K. oxytoca*, *E. coli*, *E. tarda*, *Salmonella* spp., and *A. urinae*, but negatively correlated with *R. aquatilis*, *Leuconostoc* spp., *Staphylococcus* spp. and *P. vulgaris*. Meanwhile, temperature and SO_4 showed positive correlation with *Pantoea* spp., *Vibro cholera*, *S. agalactiae*, *F. aquatile*, *P. mirabilis*, *P. fontium*, and *E. faecalis*, but negatively correlated with *D. grandis*, *A. hydrophila*, *Bacillus* spp., *A. veronii*, *Salmonella* spp. and *E. coli*.

DISCUSSION

This study represents the first effort to study the influence of water physico-chemical parameters on bacterial presence in water, sediment and fish in the peat swamp forest, paddy field and oil palm plantation in north Selangor, Malaysia. Previous studies focused mainly on peat sediment microbial community (Mishra et al., 2014; Yule,

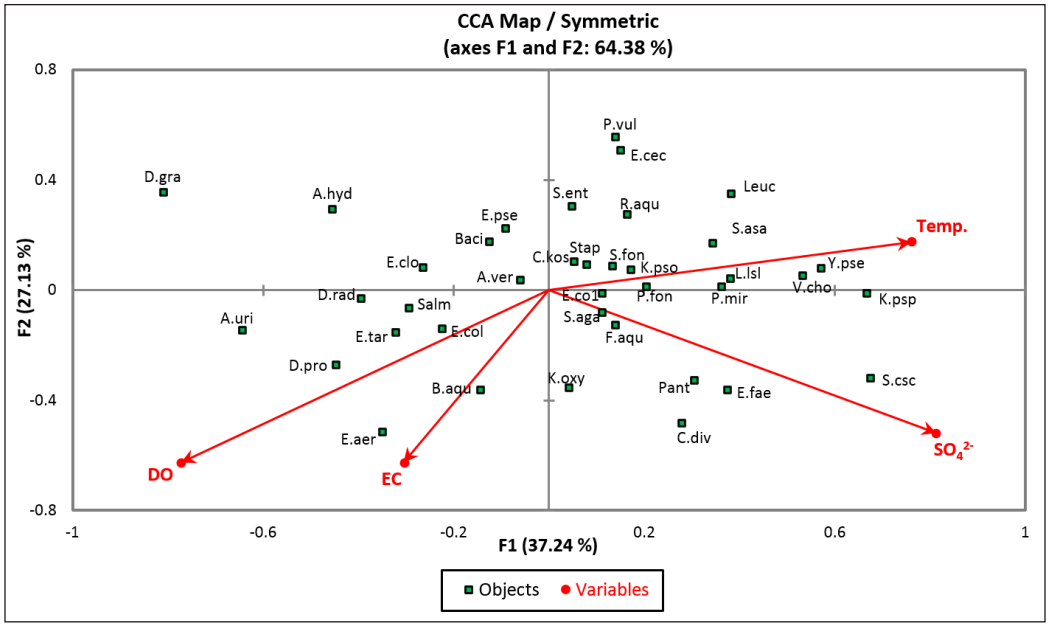

Figure 4. Canonical correspondence analysis ordination diagram showing the effect of water physico-chemical parameters on bacteria presence in oil palm plantation

2010). The bacterial load from this study was generally high, which was attributed to the relatively high ambient temperature of the aquatic systems, resulting from the direct exposure to sunlight following extensive land clearing and conversion of the natural peat forest. High ambient temperature in water bodies has been reported to support the growth of many mesophilic bacteria in natural ecosystems (Ismail et al., 2016). The bacterial load varied in the peat swamp forest, paddy field and oil palm plantation. Oil palm plantation had higher load of bacteria, ranging from 0.48×10^8 cfu mL^{-1} in water to 6.33×10^8 cfu g^{-1} in fish intestine. This was expected due to a relatively lower water quality of the oil palm plantation, resulting from waste and domestic effluent discharge as observed during the sampling time. High waste input affected the water physico-chemical parameters and provided an ideal environment for bacteria growth. It is apparent that the bacterial abundance of any water body is a direct reflection of the environmental condition due to anthropogenic disturbances (Al-Harbi & Uddin, 2003; Mishra et al., 2014). On the other hand, the peat swamp had lower bacteria load, ranging from 0.09×10^8 cfu mL^{-1} in water to 3.87×10^8 cfu g^{-1} in fish intestine. Moreover, in terms of the bacterial community structure, the bacterial diversity and richness in the peat swamp forest also showed the lowest compared to the paddy field and oil palm plantation. The lower abundance of bacteria in the peat swamp forest could be due to acidity of the habitat, which was believed to impede microbial activities (United Nations Development Programme [UNDP], 2006; Whitten et al., 2000).

The results also revealed a clear pattern variation of the bacterial load in the sediment, water, fish body surface, gill and intestine. The bacterial abundance in sediment of all habitats were significantly lower. This finding is contrary to the finding by Al-Harbi and Uddin (2003). They reported a higher abundance of bacteria in sediment compared to the water in an aquaculture pond, which might be due to high accumulation of nutrient in the pond settlement, resulting from the excessive feces of the fish and leftover fish feed. Moreover, anthropogenic disturbances and other natural events may also influence and contribute to increase the bacterial load in water (Amal et al., 2010a; Ismail et al., 2016). The bacterial loads of water and fish body surface for all habitats were not significantly different. Fish is surrounded by water, hence there is continuous interaction of the bacteria present in the water with the skin microflora. The bacteria from the water also enters the gut through the mouth and gill, thus influencing the microbial flora in the gill and intestine (Austin & Austin, 2012). However, the significantly higher bacterial load in the fish gill and intestine, compared to water may be as a result of increased metabolic activity due to the high ambient temperature of the habitats (Al-Harbi & Uddin, 2003). In addition, the bacteria input from the surrounding polluted water, the digestive tract of fish are generally colonized by assemblages

of microorganisms known as the gut microbiota. These microbiota are important in maintaining gut integrity, stepping up immunity and disease resistance, and aiding food digestion (Sullam et al., 2012). Bacterial composition in the intestines of fish are therefore not just dependent on the water in which the fish lives, but a combination of the input from the surrounding water, the gut microbiota and the dissolved organic matters that is part of the fish diet. The ingested organic matters dissolved or in suspension in the fish gut are substrate for microorganism growth, which can enhance bacterial population in the fish intestines (Zhao et al., 2012).

Higher number of bacteria isolates were recorded in the oil palm plantation and paddy field compared to peat swamp. Beside the peat characteristics impeding microbial activity in peat swamp, agricultural and domestic effluents discharged into the oil palm and paddy field enrich the water bodies, thus enhancing bacterial growth (Lee et al., 2010). Similarly, a wide range of bacterial taxa was isolated during this study. However, the most abundant species were *E. coli*, *Salmonella* spp. and *S. agalactiae*. *Escherichia coli* was a common environmental bacteria (Centers for Disease Control and Prevention [CDC], 2018), and in this study, this species was dominant in the water in all habitats and also on fish body surface and gill in the peat swamp forest, in fish gill in the paddy field, and on the fish body surface in the oil palm plantation. Besides that, *Salmonella* spp. was the dominant species in fish intestine in the peat swamp forest and on the fish surface in the paddy field. This pathogen was responsible as one of the most common foodborne infections in human (World Health Organization [WHO], 2018). Several pathogenic bacteria such as *E. faecalis*, *A. hydrophila*, *E. tarda*, *E. cloacae*, *K. pneumoniae*, *F. aquatile* and *V. cholera* were also isolated during this study. For instance, *S. agalactiae* has been reported to cause disease in cultured fish in various type of water body and also in human (Amal et al., 2008, 2010b; Chau et al., 2017), while *A. hydrophila* was responsible for tail and fin rot in fish (Dias et al., 2016), and wound infection in human (Vally et al., 2004).

Studies detailing the influence of water physico-chemical parameters and other natural events on bacterial composition in their natural habitat are well limited, as most studies are focused on fish cultured environments (Al-Harbi & Uddin, 2003, 2005). However, water physico-chemical parameters, nutrients and toxicants have been previously reported to influence bacteria density (Aisyhah et al., 2015; Gorlach-Lira et al., 2013). In this study, the peat swamp forest (conductivity, NH_3-N, temperature and Cl_2), paddy field (SO_4, NH_3-N, PO_4 and Cl_2), and oil palm plantation (DO, conductivity, SO_4 and temperature) showed varying water physico-chemical parameters that influenced the bacterial composition. However, the water conductivity, NH_3-N, temperature, Cl_2 and SO_4 consistently showed their importance in at least two of the studied habitats. The influence of temperature in this study is more

pronounced due to the high temperature, which is regarded as optimum temperature for growth of several mesophilic bacteria (Boyd & Tucker, 1998; Zamri-Saad et al., 2014). Similarly observed in this study, the NH_3-N, SO_4, PO_4, DO, EC and Cl_2 concentration in the water bodies is mostly influenced by anthropogenic chemical, nutrient and waste inputs, such as fertilizer, organic manure and domestic or industrial waste discharge (Mishra et al., 2014; Zhong et al., 2010). Thus, it was believed that high concentration, diversity and composition of bacteria from biotic and abiotic factors in the paddy field and oil palm plantation as observed in this study, reflected to the utilization of fertilizers and human anthropogenic influences. Through this inputs, dissolved organic matter becomes freely available for microbial communities, thus enhancing their growth, composition and diversity (Farrar et al., 2003; Wardle et al., 2004). Consistent with our findings, several studies reported the increasing microbial diversity as a result of increased anthropogenic inputs (Gorlach-Lira et al. 2013; Mishra et al. 2014; Zhong et al. 2010).

CONCLUSION

This study revealed that various bacteria were isolated from the water, sediment, and fish collected from the peat swamp forest, paddy field and oil palm plantation in north Selangor. In addition, the water physico-chemical parameters such as temperature, NH_3-N, Cl_2, DO, EC, SO_4 and PO_4 were all important in influencing the bacterial presence and composition in all of the studied habitats. Moreover, high bacterial load and community structure in the oil palm plantation and paddy field, compared to the peat swamp forest in indicative of pollution due to anthropogenic inputs of fertilizer, nutrients and waste in the area.

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Length-weight and Morphometric Analysis of Mud Lobster, *Thalassina anomala* from Sarawak, Malaysia

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ABSTRACT

Mud lobsters (*Thalassina* spp.) are nocturnal organisms, belong to Order Decapoda which are lesser known and least studied although their presence were widely distributed across the Indo-West Pacific Region. Mud lobster is considered as an important organism in the mangrove ecosystem for its burrowing activities and the role of its mounds or burrows as home to other animals. All mud lobster samples collected from four distinct regions in Sarawak (Kuala Tatau, Kuala Balingian, Sarikei and Lingga) were identified as *Thalassina anomala* based on the morphological characteristics. Morphometric variations between sexes of *T. anomala* were examined. Sexual dimorphism can be observed where males had significantly longer carapace length, left chelae propodus length and larger left chelae propodus width compared to females ($p<0.05$). Meanwhile, sexual dimorphism can also be seen in the abdominal width, in which it was significantly larger in females, for both absolute values ($p<0.01$) and also the relative values ($p<0.001$). The length-weight relationship of *T. anomala* were analysed between sexes by regression analysis. Results showed that males had isometric growth rate for carapace length-weight (CL/W), total length-weight (TL/W) and abdominal length-weight (ABL/W) relationships. However, females had negative allometric growth in CL/W and ABL/W relationship and isometric growth in TL/W relationship. Data from this study is able to provide baseline information

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which are useful for future reference on mud lobster resource management in Sarawak, Malaysia.

Keywords: Length-weight, morphology, morphometric, mud lobster, Sarawak, *Thalassina anomala*

INTRODUCTION

Mud lobsters (*Thalassina* spp.) are nocturnal organisms known for their burrowing activities. They are rarely seen and difficult to catch due to their habitats which are far underneath the ground. They are acknowledged as an important organism in mangrove ecosystem due to the role of their volcano-like mounds as home to other animals. The studies of mud lobster are still scarce, however eleven species of *Thalassina* were discovered distributed across Indo-West Pacific region, namely *Thalassina anomala* Herbst, 1804 (as cited in Ngoc-Ho & de Saint Laurent, 2009, p. 121), *T. emerii* Bell, 1844 (as cited in Ngoc-Ho & de Saint Laurent, 2009, p. 121), *T. gracilis* Dana, 1852 (as cited in Ngoc-Ho & de Saint Laurent, 2009, p. 121), *T. squamifera* De Man, 1915 (as cited in Ngoc-Ho & de Saint Laurent, 2009, p. 121), *T. kelanang* (Moh & Chong, 2009), *T. krempfi* (Ngoc-Ho & de Saint Laurent, 2009), *T. spinirostris* (Ngoc-Ho & de Saint Laurent, 2009), *T. spinosa* (Ngoc-Ho & de Saint Laurent, 2009), *T. australiensis* (Sakai & Türkay, 2012), *T. saetichelis* (Sakai & Türkay, 2012) and *T. pratas* (Lin et al., 2016). In Malaysia, four species of *Thalassina* have been reported which are *Thalassina anomala* by De Man (1928), *T. gracilis* by Sasekumar (1974), *T. kelanang* by Moh and Chong (2009) and *T. spinirostris* by Ngoc-Ho and de Saint Laurent (2009). *Thalassina anomala* is the most abundant species, not only in Malaysia but also in the Indo-West Pacific region (Ngoc-Ho & de Saint Laurent, 2009).

Species identification based on morphology has been described extensively in Ngoc-Ho and de Saint Laurent (2009) whilst verification of four species using morphology and molecular approach has been carried out by Moh et al. (2013). Although there are many studies on morphological characteristics of the mud lobster, most of it are focused on species differentiation.

Morphological characteristics such as body length and weight are an essential tool in scientific studies. The length-weight relationships are beneficial to understand biology and ecology of species (Kumar et al., 2018), estimation of growth rate (da Rocha et al., 2015), size at maturity (Waiho et al., 2016) and male-female differentiation (Wang et al., 2011). On the other hand, morphometric analysis has been widely conducted for studies on relative growth (Vasileva et al., 2017). This study reported on length-weight relationships and morphometric analysis of *T. anomala* collected from four locations in Sarawak, Malaysia namely Kuala Tatau (Bintulu), Kuala Balingian (Mukah), Sarikei and Lingga (Sri Aman). Given the difficulty in obtaining substantial number of organisms per sampling location, samples from all locations were pooled in order to determine length-weight relationships and morphometric analyses.

MATERIALS AND METHODS

Sample Collections

A total of 70 samples (37 males and 33 females) were purchased from local people from four distinct locations in Sarawak from March 2016 until May 2017 (Figure 1). Fifty four samples were collected from Kuala Tatau, Bintulu (3°05'42.8"N, 112°51'56.0"E), 8 samples from Kuala Balingian, Mukah (3°00'36.9"N, 112°35'26.0"E), 6 samples from Lingga, Sri Aman (1°14'01.2"N, 111°27'49.5"E) and 2 samples from Sarikei (2°07'55.5"N, 111°30'59.8"E). All samples were weighed with a digital weight and measured with a digital caliper with a precision of 0.01mm. Samples were kept in ice during transport to laboratory prior to further analyses.

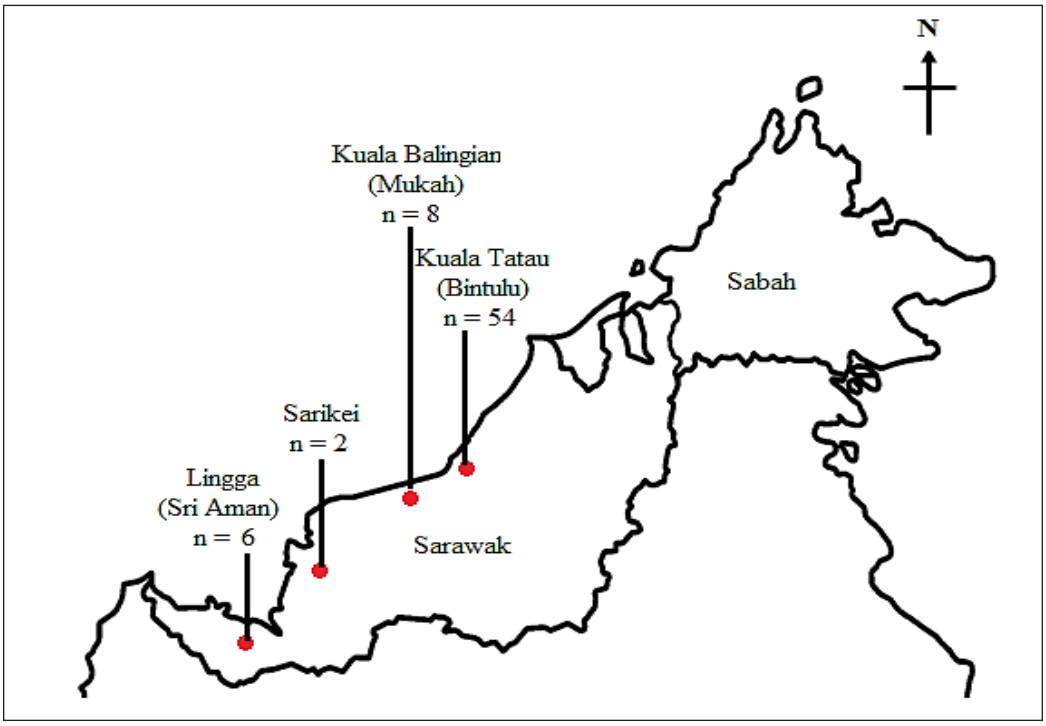

Figure 1. Sampling locations of mud lobster in Sarawak, Malaysia

Species and Sex Identification

Species identification was carried out using morphological characteristics as explain in Moh and Chong (2009) and Moh et al. (2013). Focus were made to certain features to differentiate the species such as carapace, rostrum and abdominal somite (Figure 2). Sex identification was determined according to location of the gonopores. For male, gonopores were located on inner ventral surface of coxa of pereopod 5, meanwhile for females of the pereopod 3. Females also have longer pleopods, with pleopod 2-5 being biramous bearing long setae for carrying eggs during breeding season.

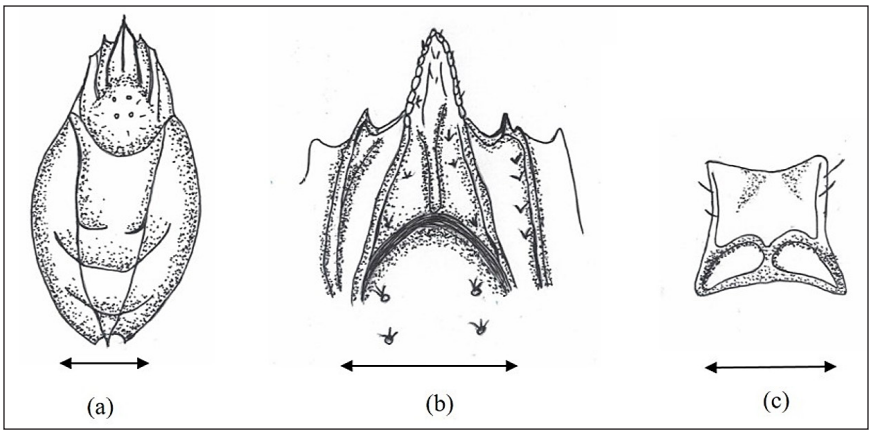

Figure 2. *Thalassina anomala*, female (TL/CL, 252.09/82.70mm). (a) = Dorsal view of carapace, (b) = Dorsal view of cephalothorax, (c) = Dorsal view of first abdominal somite. Scale bars, (a) = 30mm, (b) = 10mm, (c) = 10mm

Morphometric Analysis

Each individual sample was measured for 9 morphological characteristics; total length (TL), carapace length (CL), carapace width (CW), abdomen length (ABL), abdomen width (ABW), chelae propodus length (CPL), chelae propodus height (CPH), chelae propodus width (CPW) and wet weight (WW). Specimens with damaged or missing cheliped were not used for any propodus measurements. Figure 3a showed measurements of TL, CL, CW, ABL and ABW, while Figure 3b showed measurements of CPL, CPH and CPW.

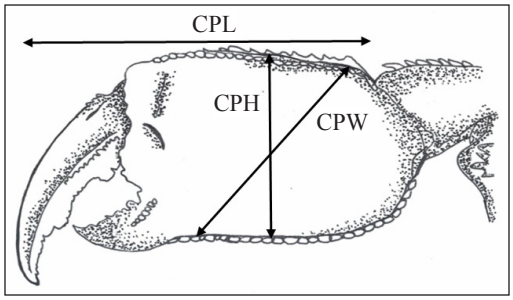

Figure 3b. Morphometric measurements of cheliped mud lobster. CPL = chelae propodus length, CPH = chelae propodus height, CPW = chelae propodus width

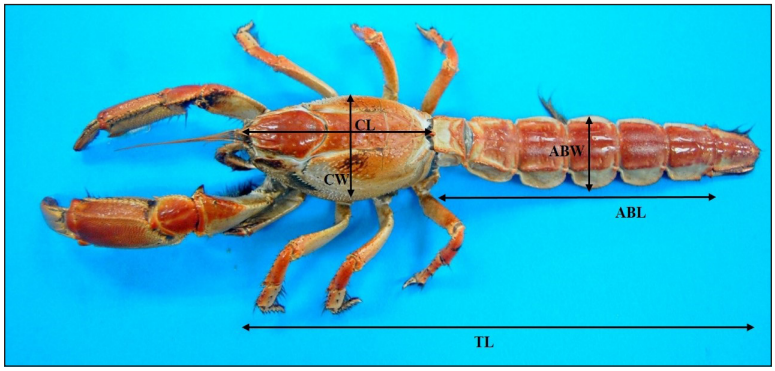

Figure 3a. Morphometric measurements of mud lobster. TL = total length, CL = carapace length, CW = carapace width, ABL = abdomen length, ABW = abdomen width

Data Analysis

The relative values of variables CL, CW, ABL, ABW, WW, CPL, CPW and CPH were calculated on the basis of their absolute values versus total length. Comparisons between sexes were analysed by means of a Student's t-test where the degree of probability was $p<0.05$*, $p<0.01$** or $p<0.001$***.

A linear regression analysis was used to analyse length-weight relationship of the specimens between sexes. The analysis was based on the allometric equation:

$$W = aL^b \quad (1)$$

(Hartnoll, 1978). The log transformed data was calculated using the formula:

$$\text{Log } W = b \log L + \log a \quad (2)$$

where a = intercept, b = slope of regression line, W = wet weight of the specimens (g) and L = total length (mm). The b values obtained from all the samples were compared using t-test to investigate allometric growth pattern of the specimens using statistic:

$$t_s = (b-3) / S_b \quad (3)$$

where t_s = t statistic value, b = slope of regression line and S_b = standard error of the slope, according to Sokal and Rohlf (1987). Isometric growth pattern can be identified when b equals to 3. Values of b greater than 3 have positive allometric growth while smaller than 3 shows negative allometric growth.

Positive allometry means weight is gaining faster than length. All statistical tests were carried out using Microsoft Excel 2013 and IBM SPSS version 20 software (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

Species Identification

Based on the morphological characteristics, all 70 samples were identified as *Thalassina anomala*. Rostrum for all samples were triangular in shape, with a shallow median sulcus (groove) that did not extend beyond the adostrals, identical to *T. anomala* and *T. squamifera*. In *T. kelanang*, the rostrum is waisted with an acute tip and a deep median sulcus (groove) that reaches behind the adostrals. In addition, tergite of first abdominal somite of all samples has two petaloid depressions in the form of an inverted V which is also in agreement with morphology of *T. anomala*. Other species such as *T. kelanang* and *T. squamifera* have an inverted Y groove instead (Moh & Chong, 2009). These morphological findings strongly suggested that mud lobster collected from all four locations are *T. anomala*.

Sexual Dimorphism

A total of 70 samples were analysed and morphometric variations between males and females were shown in Table 1. From these 70 samples, only 28 samples were analysed for cheliped measurement analysis. Relative values of carapace length of males were significantly higher than females ($p<0.05$).

Relative values for the left chelae propodus in males in terms of length and width were also significantly higher than females ($p<0.05$). Meanwhile, sexual dimorphism could also be seen in the abdominal width, in which it was significantly larger in females, for both absolute values ($p<0.01$) and also the relative values ($p<0.001$). All other parameters showed no significant difference between males and females.

Table 1
Absolute values and relative values for morphological parameters in mud lobster

Parameters	Absolute values ± SD		Relative values ± SD	
	Males (n=37)	Females (n=33)	Males (n=37)	Females (n=33)
Total Length (mm)	197.90±31.42	204.63±45.91	-	-
Carapace Length (mm)	66.02±10.66	66.06±16.16	33.36±1.30*	32.06±2.98
Carapace Width (mm)	34.92±7.14	35.88±9.68	17.54±1.50	17.34±1.93
Abdominal Length (mm)	109.68±18.25	113.43±27.40	55.38±1.62	55.14±2.89
Abdominal Width (mm)	23.09±4.06	28.63±8.96**	11.66±0.76	13.66±2.40***
Wet Weight (g)	126.67±62.03	139.46±77.27	60.86±21.29	62.78±26.34
Chelae Propodus Length (L)(mm)	60.21±14.39	55.29±17.34	29.63±4.30*	27.12±4.31
Chelae Propodus Length (R)(mm)	60.46±16.20	56.24±16.07	29.62±4.96	27.83±4.35
Chelae Propodus Width (L)(mm)	29.59±8.64	25.77±12.06	14.54±3.62*	12.15±3.99
Chelae Propodus Width (R) (mm)	28.07±10.67	24.32±9.64	13.56±4.07	11.65±3.44
Chelae Propodus Height (L)(mm)	22.44±5.83	20.00±8.30	11.19±2.72	9.75±2.89
Chelae Propodus Height (R)(mm)	21.51±8.10	19.10±6.04	10.46±3.06	9.49±2.24

For all propodus parameters, n= 28 for males and n = 28 for females. L = left, R = right. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

The results of this study are in agreement with many morphometric studies of crustaceans. It is common for males to have longer carapace length and bigger chelae (Vasileva et al., 2017; Zaikov et al., 2011). Body size and chelae are among the important morphological characteristics for better opportunities for competition, predation and reproduction (McLain & Pratt, 2007; Miyajima et al., 2012).

According to Rufino et al. (2004), males crab (*Liocarcinus depurator*) had broader carapace than females. Larger carapace is crucial for the insertion of muscles of poreiopods and chelipeds which may contribute to stronger muscles for defence against predators. Besides, larger carapace in males is useful during agonistic interactions in the competition for females (Moore, 2007; Reid et al., 1994).

In this study, females appeared to have larger abdominal width than males. The sexual dimorphism in the abdomen size between sexes of *T. anomala* might be due to the differences in the role of the male gonopods and female pleopods. In females, the increase in abdomen size with long setae is necessary as an incubation chamber for egg development. Daniels (2001) reported that in freshwater crab (*Potamonautes*

warren), females have marked increase in abdomen size as well for the purpose of carrying eggs during breeding season. According to Goldstein et al. (2014), female lobsters (*Homarus americanus*) have larger abdominal areas to allow attachment of eggs to ovigeorus setae of pleopods on the ventral side of their abdomen.

In decapod crustaceans, cheliped also known as the first pereiopod with a chela (claw) formed by a modification of the dactylus and propodus (Claverie & Smith, 2010). The chelipeds in decapods have important role in feeding, territory defense, for facilitate predation and for communication during courtship and mating (Mariappan et al., 2000). According to Pillai (1990) and Voris and Murphy (2002), the chelipeds of mud lobster are mainly used for burrowing, combat or defence against predators and for feeding. The chelipeds are variable in sizes and no specific handedness was observed in *T. anomala*. Males are more prominent, having larger cheliped than females and sexual dimorphism in terms of cheliped size is common in decapod crustacean (Hartnoll, 1978). Previous study done by Claverie and Smith (2009) revealed sexual dimorphism in cheliped length of the squat lobster (*Munida rugose*). In addition, sexual dimorphism in cheliped size can also be observed in the temperate crayfish, (*Orconectes rusticu*s) (Snedden, 1990). In the present study, *T. anomala* exhibits sexual dimorphism where males had significantly longer left chelae propodus length and larger left chelae propodus width than females. Greater cheliped size in males is expected since cheliped plays an important role during agonistic interactions and used as a weapon against predators (Claverie & Smith, 2007) or for sexual competition (Mariappan et al., 2000). Both males and females *T. anomala* in this study have monomorphic and dimorphic chelipeds, with no significant preferences between sexes.

Length-weight Relationship

The total length-weight relationships showed an isometric growth pattern for both sexes and combined sexes as recorded in Table 2. For males ($b = 3.292$, $p > 0.05$) while females ($b = 2.928$, $p > 0.05$) and combined sexes ($b = 3.023$, $p > 0.05$). The regression equation for TL/W relationship for males, females and combined sexes were as follows:

$$\text{Log } W = 3.292 \text{Log } TL - 5.495 \quad (R^2 = 0.962) \tag{4a}$$

$$\text{Log } W = 2.928 \text{LogTL} - 4.680 \quad (R^2 = 0.965) \tag{4b}$$

$$\text{Log } W = 3.023 \text{Log } TL - 4.890 \quad (R^2 = 0.960) \tag{4c}$$

The scatterplots of the TL/W relationship for males and females were plotted in Figures 4a and 4b.

The regression analysis of carapace length-weight relationship of *T. anomala* is shown in Table 3. Males had isometric growth ($b = 3.138$, $p > 0.05$) while females had negative allometric growth ($b = 2.740$, $p < 0.05$). For combined sexes, negative allometric growth was observed ($b = 2.822$,

Table 2
Descriptive statistics and estimated parameters of total length-weight relationship of Thalassina anomala *for both sexes in Sarawak, Malaysia*

Sex	N	Total length (mm)		Wet weight (g)		Regression parameters					Allo-metric growth
		Min	Max	Min	Max	Log a	b	SE	CL	R^2	
M	37	144.27	252.55	47.00	241.00	- 5.495	3.292	0.116	3.055- 3.528	0.962	I
F	33	98.50	265.27	15.00	287.00	- 4.680	2.928	0.099	2.724- 3.131	0.965	I
C	70	98.50	265.27	15.00	287.00	- 4.890	3.023	0.077	2.870- 3.177	0.960	I

M = male, F = female, C = combine sexes, N = number of specimens, Min = minimum, Max = maximum, a = intercept, b = slope, SE = standard error of b, CL = confidence limits of b, R^2 = coefficient of determination, I = Isometric growth

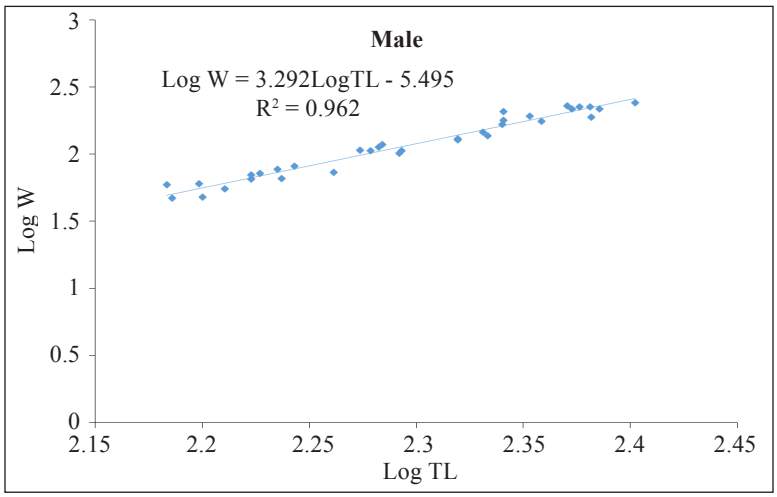

Figure 4a. Total length-weight relationship of male *Thalassina anomala* in Sarawak, Malaysia. W = wet weight, TL = total length

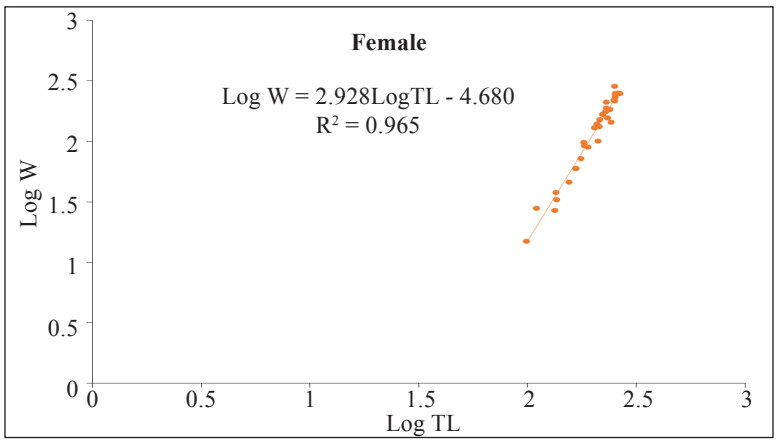

Figure 4b. Total length-weight relationship of female *Thalassina anomala* in Sarawak, Malaysia. W = wet weight, TL = total length

$p < 0.05$). The regression equation for CL/W relationship for males, females and combined sexes were as follows:

$$\text{Log } W = 3.138\text{LogCL} - 3.650 \quad (R^2 = 0.964) \quad (5a)$$

$$\text{Log } W = 2.740\text{LogCL} - 2.894 \quad (R^2 = 0.950) \quad (5b)$$

$$\text{Log } W = 2.822\text{Log CL} - 3.057 \quad (R^2 = 0.950) \quad (5c)$$

The scatterplots of the CL/W relationship for males and females were plotted in Figures 5a and 5b.

Table 3
Descriptive statistics and estimated parameters of carapace length-weight relationship of Thalassina anomala *for both sexes in Sarawak, Malaysia*

Sex	N	Carapace length (mm)		Wet weight (g)		Regression parameters					Allo-metric growth
		Min	Max	Min	Max	Log a	b	SE	CL	R^2	
M	37	42.53	85.13	47.00	241.00	-3.650	3.138	0.121	2.887-3.388	0.964	I
F	33	19.10	83.54	15.00	287.00	- 2.894	2.740	0.127	2.478- 3.002	0.950	-A
C	70	19.10	85.13	15.00	287.00	- 3.057	2.822	0.093	2.635- 3.009	0.950	-A

M = male, F = female, C = combine sexes, N = number of specimens, Min = minimum, Max = maximum, a = intercept, b = slope, SE = standard error of b, CL = confidence limits of b, R^2 = coefficient of determination, I = Isometric growth, -A = negative allometric growth

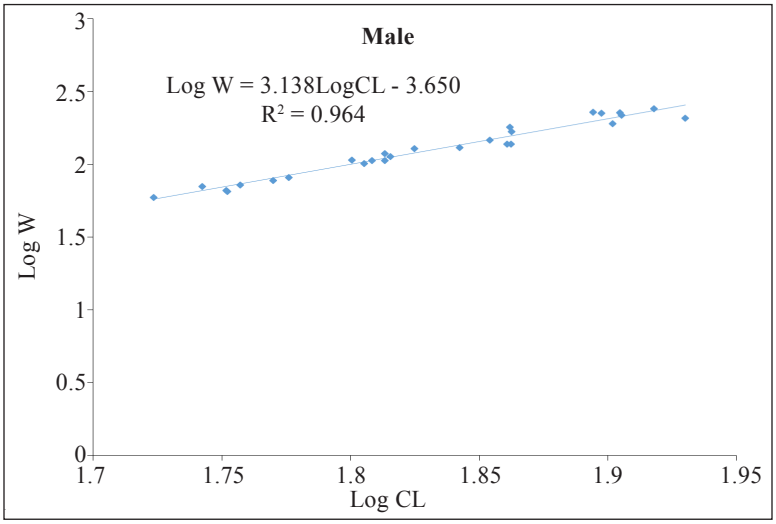

Figure 5a. Carapace length-weight relationship of male *Thalassina anomala* in Sarawak, Malaysia. W = wet weight, CL= carapace length

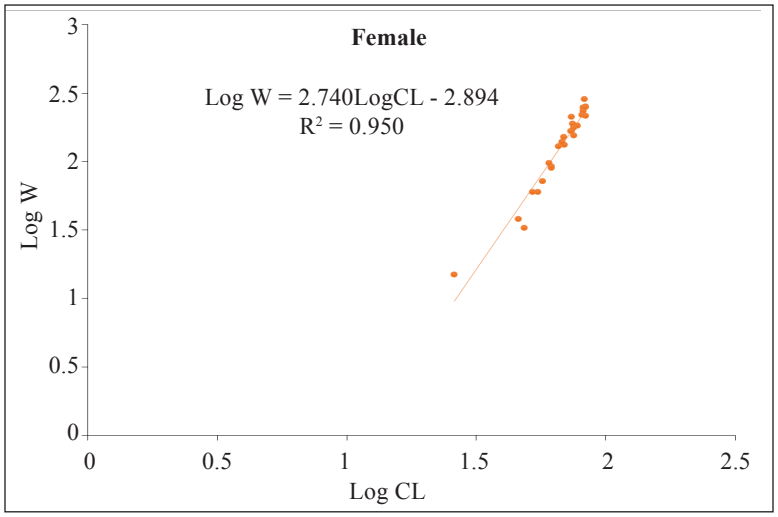

Figure 5b. Carapace length-weight relationship of female *Thalassina anomala* in Sarawak, Malaysia. W = wet weight, CL = carapace length

As shown in Table 4, the abdominal length-weight relationship showed an isometric growth in males (b = 2.981, p > 0.05) and negative allometric growth in females (b = 2.743, p < 0.05) and combined sexes (b = 2.821, p < 0.05). The regression equation for ABL/W relationship for males, females and combined sexes were as follows:

$$\text{Log W} = 2.981\text{Log ABL} - 4.016 \quad (R^2 = 0.956) \quad (6a)$$

$$\text{Log W} = 2.743\text{Log ABL} - 3.547 \quad (R^2 = 0.959) \quad (6b)$$

$$\text{Log W} = 2.821\text{Log ABL} - 3.699 \quad (R^2 = 0.955) \quad (6c)$$

The scatterplots of the ABL/W relationship for males and females were plotted in Figures 6a and 6b. All regression models were statistically significant (p < 0.05).

Table 4
Descriptive statistics and estimated parameters of abdominal length-weight relationship of Thalassina anomala *for both sexes in Sarawak, Malaysia*

Sex	N	Abdominal length (mm)		Wet weight (g)		Regression parameters					Allometric growth
		Min	Max	Min	Max	Log a	b	SE	CL	R^2	
M	37	75.65	140.46	47.00	241.00	- 4.016	2.981	0.108	2.762- 3.201	0.956	I
F	33	40.64	151.21	15.00	287.00	- 3.547	2.743	0.106	2.526- 2.959	0.959	-A
C	70	40.64	151.21	15.00	287.00	- 3.699	2.821	0.076	2.670- 2.972	0.955	-A

M = male, F = female, C = combine sexes, N = number of specimens, Min = minimum, Max = maximum, a = intercept, b = slope, SE = standard error of b, CL = confidence limits of b, R^2 = coefficient of determination, I = Isometric growth, -A = negative allometric growth

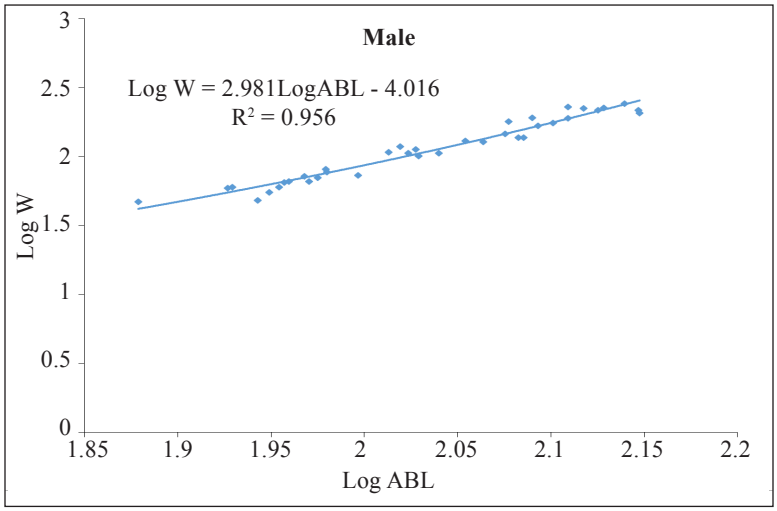

Figure 6a. Abdominal length-weight relationship of male *Thalassina anomala* in Sarawak, Malaysia. W = wet weight, ABL = abdominal length

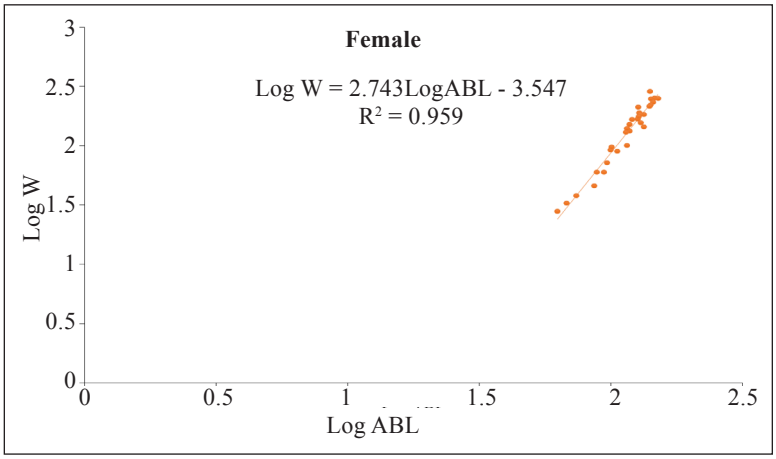

Figure 6b. Abdominal length-weight relationship of female *Thalassina anomala* in Sarawak, Malaysia. W = wet weight, ABL = abdominal length

The present study showed the differences of growth pattern between sexes of *T. anomala*. There are several factors that could contribute to the variations in allometry of decapod crustaceans such as age, sex, differences in diet, foraging behaviour, availability and quality of food, environmental changes regulated by abiotic factors which include season, temperature, salinity and rainfall (Moutopoulos & Stergiou, 2002; Robertson & Butler, 2003; Taddei et al., 2017). In morphometric relationship of length and weight of crustaceans, analysis of total length, carapace length and abdominal length in length-weight relationship are prevalent as these parameters are less variables and more easily measured in the field. As such, the

use of these measurements in aquaculture are highly recommended because there are the most precise and simple alternative of analysing growth pattern of organism (Lalrinsanga et al., 2012).

In this study, *T. anomala* males had isometric growth in TL/W, CL/W and ABL/W relationships, indicating that total length, carapace length, abdominal length and body weight of males were growing at the same rate.

Growing at slower rate may be advantageous in terms of energy efficiency as larger individuals require more energy and food intake, which lead to more foraging, thus increase risk of predation. Food limitation and availability may also influence growth rate. According to Claverie and Smith (2009) and Silva et al. (2014), crustacean males are more likely to experience change in energy allocation, in which males may investing more energy in cheliped growth compare to other parts of their body, since cheliped mostly used for agonistic interaction and as an anti-predator adaptation. Meanwhile, *T. anomala* females had negative allometric growth in CL/W and ABL/W relationship, indicating that carapace length and abdominal length increased at a faster rate than body weight. In addition, females had isometric growth in TL/W relationship, where total length increased at a rate proportional to body weight. This growth pattern are presumably a result of devoting and investing more energy in reproduction over growth by females (Robertson & Butler, 2003; Silva et al., 2014). The abdomen part used as an incubation chamber for developing eggs, hence the abdomen growth rate are faster to effectively carry all the eggs produced by the females.

Similar growth patterns can be observed in previous study done by Senevirathna et al. (2014) which showed that in spiny lobster (*Panulirus Homarus*), isometric growth was observed for males in TL/W relationship and negative allometric growth for females in CL/W relationship.

CONCLUSION

This study reported that all mud lobster samples collected from Kuala Tatau (Bintulu), Kuala Balingian (Mukah), Sarikei and Lingga (Sri Aman) are from the same species which is *T. anomala*. Morphometric analyses showed that sexual dimorphism occurred in the carapace length, abdominal width, chelae propodus length and chelae propodus width. The present study also provides basic information of length-weight relationships of *T. anomala* which is useful as references of their biomass and specific management units for their conservation in the future.

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Heavy Metals Concentrations in Stormwater and Tilapia Fish (*Oreochromis Niloticus*) in Kuala Lumpur Holding and Storage SMART Ponds

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ABSTRACT

The issue of heavy metal contamination in urban stormwater has become a major concern for environmental pollution control agencies worldwide due to toxic effects on aquatic organisms and human health. The aim of this study is to determine the levels of heavy metals (Pb, Cd, Cu, Cr and Zn) in surface stormwater and tilapia fish (*Oreochromis niloticus*) obtained from holding and storage ponds of the Stormwater Management and Road Tunnel (SMART) Project in Kuala Lumpur, Malaysia. Results have indicated that the concentrations of all heavy metals in stormwater were lower than the recommended water quality criteria established by the United States Environmental Protection Agency (US EPA). On the other hand, the concentrations of Cd, Cu, Pb and Zn detected in fish were below threshold values suggested by the Food and Agriculture Organization (FAO) and Malaysian Food Regulations (MFR) standards. Only Cr was much higher than FAO limits for fish consumption. Additionally, the content of Cr, Pb and Zn was higher in fish samples collected from holding pond compared to those in storage pond. As the study reflects that Cr in tilapia fish from SMART ponds is of a

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high concentration, certain harmful effects on human health may result. Accordingly, the research recommends avoiding fishing from ponds, as they accumulate a significant amount of waste consumed by the fish.

Keywords: Heavy metals, *Oreochromis niloticus*, SMART ponds, stormwater ponds, tilapia fish

INTRODUCTION

Rapid urbanization and daily human activities are significant sources of pollutants that affect the quality of urban stormwater. Due to their direct adverse effects on the receiving waters and also on human health, environmental pollution control agencies worldwide have become greatly concerned about concentrations of heavy metals in urban stormwater (Joshi & Balasubramanian, 2010). As stormwater flows over different impermeable surfaces, it picks up and transports pollutants such as heavy metals, nutrients, and organic chemicals and discharges them directly to local receiving water bodies (Brown & Peake, 2006). It could be considered that the concentrations of heavy metals in stormwater can be controlled by different factors such as climate, nature of fuels used, the type of vehicles, average daily traffic, etc. (Leung & Jiao, 2006). The majority of heavy metals which penetrate the various bodies of water are normally associated with particulates and, as a result of settling time, they accumulate in the bottom sediments of the receiving water. Therefore, they become available for bioaccumulation in organism life in the ponds (Stead-Dexter & Ward, 2004; Stephansen et al., 2012).

As a matter of fact, fish is situated at the top of the aquatic food chain. As a result, great amounts of heavy metals can normally be accumulated from water, food, and sediments (Yılmaz et al., 2007). What makes the problem worse is the fact that fish consumption is a major route of chemical exposure for human beings (Ikem et al., 2003). Thus, the fish needs to be carefully examined to ensure that unnecessary high levels of toxic metals are not being transferred to humans through fish consumption. However, fish is also an important food resource for human beings worldwide, providing a beneficial source of high quality proteins, minerals and vitamins (Chen et al., 2013).

In urban stormwater management, stormwater ponds are widely used to provide protection of downstream areas from flooding through temporary storage of stormwater runoff from impermeable surfaces. Moreover, stormwater ponds improve the quality of stormwater typically by removal of suspended solids and associated pollutants (Krishnappan & Marsalek, 2002). The SMART Project is designed primarily to reduce the stormflow from the upper Klang/Ampang catchment area away from the Kuala Lumpur city centre to a manageable quantity within the capacity of the Klang River.

Since the SMART Project began operations in 2007 (Khalil et al., 2011), the stormwater wastes in the SMART ponds have a high chance for accumulation to an extent that exceeds permissible water quality standards. It is hypothesized that

resident organisms will be implicated by the accumulation of metals and adversely-beneficial compounds. Therefore, the ultimate objective of this study is to determine the concentrations of heavy metals, specifically Cd, Cr, Cu, Pb, and Zn in surface stormwater, as well as in tilapia fish (*O. niloticus*) of the SMART ponds receiving stormwater runoff from different land use areas in the Ampang/ Klang catchment.

MATERIALS AND METHODS

Site Description

The stormwater management ponds were constructed as a part of the SMART project, which is part of the overall Kuala Lumpur Flood Mitigation System. The SMART project mainly consists of two ponds namely, the holding pond and the storage pond. Both of these are connected to the SMART tunnel. The holding pond was designed to retain excess floodwater diverted from the confluence of the Klang and Ampang Rivers during major storm events when the total discharge in the rivers' confluence exceeds 70 m^3/s. The diverted floodwater is temporarily held until it reaches a certain permitted level before being released through the SMART Tunnel (bypass tunnel) to a storage pond (Dominic et al., 2016). The pond is located in Kampung Berembang, (3°09'51.8"N, 101°44'35.6"E) with an area of 8 hectares having a capacity of 0.6 million m^3. On the other hand, the storage pond is situated in Taman Desa, (3°06'08.4"N, 101°41'31.5"E) with an area of 23 hectares and a capacity of 1.4 million m^3. The catchments area is approximately 121 km^2 consisting of parking areas, building roofs and a bridge in addition to motorways. The primary sources of pollution in the study area are motor vehicle emissions, vehicle tyre wear, road asphalt, building materials and atmospheric deposition. According to meteorological measurements taken between 2014 and 2017 (provided by the Malaysian Meteorological Services Department), the average annual precipitation in the study area is 2790.7 mm. The annual mean temperature is 27°C with the monthly mean temperature varying from maximum 32°C to minimum 23°C (Dominic et al., 2015).

Sample Collection

The stormwater and fish samples were collected in November 2016 (approximately one month after diverting stormwater flow to SMART ponds). In each pond, two stations closer to the centre of the pond were selected for surface stormwater sampling. The samples were obtained from 50-cm depth in triplicate from four stations named as HPS1, HPS2, SPS1 and SPS2. The samples were collected in acid washed polyethylene bottles, kept in an ice box, and transported to the laboratory for the further analysis. Tilapia fish (*Oreochromis niloticus*) samples were caught randomly over the ponds in triplicate, washed with distilled water, kept in separate polythene bags, placed in ice-cooled containers and immediately transported to the laboratory. Upon arrival at the laboratory, samples were washed with ultrapure water, weighed, measured by total

length and kept frozen at -20ºC (El-Moselhy et al., 2014; Mendil et al., 2010). The fish samples ranged from 22–25 cm in total length and 170–280 g in body weight.

Sample Preparation and Analysis

Stormwater sampling and analytical techniques were performed in accordance with APHA Standard Methods (American Public Health Association [APHA], 2005). Briefly, about 100 mL of each of the stormwater samples were passed through a 0.45 mm cellulose nitrate membrane filter. The filtrate samples were therefore acidified to pH < 2 using ultrapure nitric acid (ACS Reagent, 70%). The samples were then refrigerated at 4ºC before analysis. The fish samples were dissected and washed with double distilled water (Malik et al., 2010). Dorsal muscle samples were taken, homogenized and weighed for the metal analysis. Individual samples were oven-dried at 80ºC for 48 h in acid-washed petri dishes and were ground to a fine powder. Approximately 0.5 g dry weight of powdered muscle was digested in a 10 ml of concentrated nitric acid (R&M Grade 69%). Samples were heated first at low temperature (40°C) for 1 h and then at 140°C for at least 3 h (Yap et al., 2002). Digested samples were filtered through pre-washed 0.45 µm cellulose nitrate membrane filter (Alam et al., 2002). The samples were then diluted with ultrapure water to 50 mL and preserved at 5ºC until further analysis (Bashir et al., 2011). Blanks were used simultaneously in each batch of analysis. The heavy metal analyses were conducted using A Perkin-Elmer ELAN DRC-e Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Canada).

Reagents and Standards

All reagents used during analysis were of analytical reagent grade. Ultrapure water (Milli-Q System, Millipore) was used for all dilutions. Ultrapure quality nitric acid (HNO_3) (R&M Grade 69%) was used for digestion of fish samples. The suitable standard solutions for the calibration were prepared by dilution of the stock solution of 10 mg/L. All the plastic and glassware were cleaned by soaking in dilute HNO_3 and rinsed several times with ultrapure water prior to use. The quality of the analytical procedures was checked with a Certified Reference Material DORM-4, fish protein for trace metals (NRCC, Canada) (Table 1).

Table 1
Observed and certified values for the elements in standard reference material (Dorm-4) (µg g^{-1} dry weight)

Element	Observed value **(mean ± SD)**	Certified value	Recovery (%)
Cd	**0.266±0.001**	**0.299 ± 0.018**	**89**
Cr	**2.031±0.021**	**1.87 ± 0.18**	**109**
Cu	**15.85±0.373**	**15.7 ± 0.46**	**101**
Pb	**0.394±0.003**	**0.404 ± 0.062**	**98**
Zn	**47.92±01.32**	**51.6 ± 2.8**	**93**

Statistical Analyses

Results of heavy metal concentrations were analysed using SPSS software 23. Independent t-test at 95% was used to determine the significant differences of all heavy metals investigated in stormwater and fish between holding and storage SMART ponds ($P < 0.05$).

RESULTS AND DISCUSSION

Heavy Metal Concentrations in Stormwater

The concentrations (µg/l) of heavy metals in stormwater of SMART ponds are presented in Table 2. The study results revealed that the concentration levels of heavy metals (Cr, Cd, Pb and Zi) in holding pond were higher than those in storage pond; while Cu was found to be the opposite being higher in a storage pond. The reason for that might be due to the fact that a holding pond was the first recipient for urban stormwater runoff which came into contact with debris and various pollutants. Both SMART ponds exhibited small decreases in metal concentrations between the stations in each pond. This could be due to different heavy metals exhibiting different behaviours. For instance, Zn, Cu, and Cd are found to be associated with the dissolved phase (e.g. colloidal material), (Huber et al., 2016), while Pb is strongly associated and bound to particles and Cr is relatively bound to organic matter (Karlsson et al., 2010).

On the level of stations, the concentration of Cr ranged from 9.8 µg/L at SPS1 to 15.8 µg/L at HPS1. On the ponds level, the mean values of Cr were 14.7 µg/L and 10.7 µg/L for holding pond (HP) and storage pond (SP) respectively. In urban stormwater runoff, the major source of Cr is the body surface of vehicles, which is coated with hexavalent Cr for corrosion prevention (Aryal et al., 2010). There was a significant difference in the mean of Cr level between the ponds (p=0.001, t=5, df=10) (Table 3). The mean values of Cr in the stormwater of both ponds were found to be lower than the acute criteria (CMC) (570 µg/l) and chronic criteria (CCC) (74 µg/l) allowed by United States Environmental Protection Agency (US EPA, 2017).

The highest concentration of Cu in stormwater samples was measured in SPS2 and was found to be 3.69 µg/L; while its lowest concentration was measured in HPS1 reaching 2.75 µg/L. On the part of ponds, the mean concentration of Cu in stormwater of SP was slightly higher compared to its concentration in HP (3.5 µg/L and 3.08 µg/L respectively). This could be attributed to motorway dust from the SMART Tunnel that washes off to SP during diversion of the excess stormwater runoff from HP. Statistical analysis using independent t-test indicates no significant difference results of the Cu levels between the ponds (p=0.06, t= -2.2, df=10). The Cu contamination in urban stormwater is associated with specifically: the intensity of vehicular traffic (Gunawardena et al., 2013); vehicle brake emissions (Davis et al., 2001) and corrosion of building materials (e.g., roofs, pipes, etc.) (Göbel et al., 2007). Cu values were far below threshold levels of US EPA guidelines CCC (9 µg/l) and CMC (13 µg/l).

The mean value of Zn was 19.63 µg/l in stormwater samples collected from HP, and 15 µg/l for samples collected from SP. On the level of stations, the concentrations of Zn ranged from 14.98 at SPS2 to 20.4 µg/l at HPS2. The results revealed that there was a significant difference in the mean of Zn levels between the ponds (p<0.001, t=7, df=9). According to the study results, the levels of Zn in both ponds were lower than permissible standard levels of US EPA (120 µg/l). It could be mentioned that the concentrations of Zn are varied in the runoff of traffic area compared with other heavy metals due to its existence in crumbs of car tyre rubber and other galvanized structures (Huber et al., 2016). In urban areas, galvanized metals and tyre wear are the two greatest sources of zinc contamination (Vos & Janssen, 2009).

Cd and Pb at the two studied ponds were found to have concentrations lower than the permissible limits of the US EPA guidelines (0.72-1.8 µg/l and 2.5-65 µg/l respectively). The level of Cd in stormwater samples ranged from 0.03 µg/l at SPS1 to 0.09 µg/l at HPS1. From the perspective of ponds, the mean value of Cd in HP 0.07 µg/l was slightly higher than that in SP, 0.05 µg/l. The results revealed no significant difference in the mean of Cd levels between the ponds (p=0.13, t=1.7, df=10). On the other hand, the mean of Pb values in both the ponds were significantly different (p<0.001, t=11, df=10). In urban runoff, the major contributors for Cd contamination are building walls and atmospheric deposition (Davis et al., 2001), while vehicles are the largest source of lead contamination in the urban environment. According to Hwang et al. (2016), lead has been added to gasoline as well as many vehicle parts, including wheel rims, batteries, aluminium alloys and wheel balancing weights.

Table 2
Heavy metal concentrations (µg/L) in surface stormwater of SMART ponds

Location	Cd mean ± SE	Cr mean ± SE	Cu mean ± SE	Pb mean ± SE	Zn mean ± SE
Holding Pond (HP)					
HPS1[a]	0.09±0.00	15.8±0.9	2.75±0.06	1.92±0.02	18.77±0.7
HPS2[b]	0.05±0.00	13.5±0.6	3.41±0.13	2.38±0.02	20.43±0.6
Total mean	0.07±0.03	14.67±1.7	3.08±0.4	2.15±0.3	19.63±1.3
Storage Pond (SP)					
SPS1[c]	0.03±0.00	9.8±0.2	3.31±0.06	0.69±0.02	15.08±0.6
SPS2[d]	0.07±0.00	11.6±0.4	3.69±0.07	0.98±0.03	14.98±0.6
Total mean	0.047±0.02	10.7±1.1	3.5±0.23	0.83±0.16	15.0±0.9
USEPA-(CMC)[e]	1.8	570	13	65	120
USEPA-(CCC)[f]	0.72	74	9.0	2.5	120

[a] holding pond station1, [b] holding pond station2, [c] storage pond station1, [d] storage pond station2
[e] CMC: criterion maximum concentration (acute effect), [f] CCC criterion continuous concentration (chronic effect)

Table 3
Independent t-test differences between heavy metals concentrations in stormwater of SMART ponds

	F	t	df	Sig. (*P*-value)
Cd	4.928	1.7	10	0.13
Cr	0.51	4.8	10	0.001
Cu	3.4	-2.2	10	0.06
Pb	22.2	11	10	0.000
Zn	0.13	7	9	0.000

Independent t-test at 95% significance: ($P < 0.05$)

Heavy Metal Concentrations in Fish

Heavy metals can enter fish via five main pathways (water, gills, skin, food and non-food particles), mixed with blood, and are carried to either a storage point or to the liver for its storage or transformation (Shinn et al., 2009). Heavy metal accumulation in fish tissues depends not only on concentrations in the environment but also on many geochemical and biological factors that influence the bioavailability of metals, such as: fish size; temperature; pH; and organic ligands (Camusso et al., 1995). In tilapia fish, the concentrations of heavy metals (Zn, Cu, Pb, Cr and Cd) collected from SMART ponds are given in Table 4. As can be seen in Table 5, independent t-test analysis shows that all heavy metal concentrations were significantly different between HP and SP ($p < 0.001$, df = 4). Cr levels in analysed fish obtained from HP were higher (22.03 µg/g) than those in SP (19.1 µg/g). Moreover, the mean of Cr values from both ponds were higher than the levels recommended by Food and Agriculture Organization of the United States (FAO, 1983) (2.0 µg/g), and much higher than those reported from Ampang Hilir Lake (Said et al., 2012) (2.15 µg/g). It has been reported that Cr (III) is a fundamental nutrient that potentiates the action of insulin and hence it affects fat, protein and glucose metabolism (Taghipour & Azizi, 2010). Conversely, Cr (VI) is a well-known human carcinogen (Tuzen & Soylak, 2007).

In the same vein, Zn is an important element in human nutrition which fulfils several biochemical functions in human metabolism. However, an excessive intake of Zn can cause critical adverse effects (Scherz & Kirchhoff, 2006). With regard to HP tilapia fish, the content of Zn was found to be slightly higher (15.39 µg/g) than that in SP (12.34 µg/g). Furthermore, the concentrations of Zn in both ponds for tilapia fish were far below the threshold values established by FAO (1983) (30 µg/g) and Malaysian Food Regulation (MFR, 1985) MFR (100 µg/g). The results show that Zn values are lower than those reported on UPM Pond (Yap et al., 2015) (15.70 µg/g). Conversely, Zn achieved higher values compared to those reported on Ampang Hilir Lake (1.86 µg/g).

Cu is also an essential element which plays a vital role in biological systems. Nevertheless, high intake of Cu can cause adverse health risks (Sivaperumal et al., 2007). The content of Cu in muscles of fish samples from SP was found to be slightly higher (0.77 µg/g) than those collected from HP (0.65 µg/g). However, Cu levels in the SMART ponds were lower than the maximum permitted concentrations

proposed by FAO and MFR (10 µg/g and 30 µg/g respectively). They were also found to be lower than the results obtained by previous studies on Ampang Hilir Lake, and UPM ponds (4.06 µg/g and 1.42 µg/g respectively).

Pb and Cd are known as toxic elements that up until recently have had an unknown biological function. They have been shown to have carcinogenic consequences on humans and aquatic biota (Malik et al., 2010). In this study, the concentration level of Cd in fish samples was extremely low, 0.003 µg/g at SP and slightly lower at HP 0.002 µg/g. However, Cd absorption constitutes a serious risk to humans, since it may cause reproductive deficiencies, kidney diseases and skeletal damage (Järup, 2003). With regard to Pb, the levels of concentration were 0.23 and 0.19 µg/g for analysed fish samples in both HP and SP respectively. It has been reported that the effects of lead in the body are characterized by decline in both intellectual and cognitive growth in children. Similarly, it increases cardiovascular diseases and blood pressure irregularity in adults Commissions of the European Communities (COEC, 2001). The levels of Cd and Pb in tilapia fish in the two ponds were lower than those obtained in the studies mentioned above, and below the maximum lead level permitted by the FAO and MFR, as shown in Table 4.

Overall, Cr, Pb and Zn in tilapia fish caught from HP were found to have concentrations higher than those collected from SP. This could be due to two main reasons. First: HP is the initial recipient for pollution loads in stormwater runoff that come from different land uses in the urban catchment of Ampang-Klang. Second: urban stormwater runoff diverted to HP is often held at least for a period of 6 hours before being released to the SP via the SMART tunnel. Thus, sediment and other stormwater runoff pollutant loads find enough time to be settled down in the bottom of HP stormwater body. Over time, such pollutants as heavy metals become available for bioaccumulation in tilapia fish of HP.

In contrast, Cd and Cu in fish samples showed slightly higher concentrations in SP compared to those in HP. In fact, there are two possibilities behind these high levels. The first might be attributed to the SMART motorway tunnel dust resulting from traffic activities (an average of 25,000 vehicles per day pass through the motorway tunnel). This dust is washed off by stormwater diverted from HP through the SMART tunnel and subsequently discharged into SP. The second possibility might be due to the atmospheric pollutant deposition as an important source for Cd and Cu contamination (Davis et al., 2001). It is worth mentioning here that SP is very far from any urban or industrial activities and, thus, their pollution.

The high concentrations of Cr detected in the muscles of tilapia fish in the SMART ponds renders these fish unsafe for human consumption, Therefore, it could be reported that local community members are not encouraged to have fish, obtained directly from the ponds or indirectly from the Kerayong River when excess stormwater is diverted back from ponds to the river during heavy rainfall events in the Klang/ Ampang areas.

Table 4
Heavy metal concentrations (μg/g dry wt) in the muscles of tilapia fish from SMART ponds

Location	**Cd** mean ± SE	**Cr** mean ± SE	**Cu** mean ± SE	**Pb** mean ± SE	**Zn** mean ± SE	References
HP	0.002 ± 0.00	22.03 ± 0.2	0.65 ± 0.00	0.23 ± 0.00	15.39 ± 0.1	This study
SP	0.003 ± 0.00	19.1 ± 0.2	0.77 ± 0.01	0.19 ± 0.00	12.34 ± 0.1	
Ampang Hilir Lake	0.18	2.15	4.06	0.97	1.86	(Said et al., 2012)
UPM Pond	0.84	-	1.42	1.64	15.70	(Yap et al., 2015)
	-	2.0	10	4	30	FAO (1983)
	1.0	-	30	2.0	100	MFR (1985)

Table 5
Independent t-test differences of heavy metals concentrations in tilapia fish from SMART ponds

	F	t	df	Sig. (*P*-value)
Cd	1.535	-25.86	4	0.000
Cr	0.009	10.31	4	0.000
Cu	0.297	-16.31	4	0.000
Pb	0.672	53.94	4	0.000
Zn	0.101	10.84	4	0.000

Independent t-test at 95% significance: ($P < 0.05$)

CONCLUSION

The results of this study demonstrated that the studied heavy metal concentrations in the stormwater of SMART ponds (holding and storage ponds) are found to be lower than the water quality criteria proposed by USEPA. According to the study results, the concentrations of Cr, Cd, Pb and Zi in stormwater samples of HP were found to be higher than those in SP except for Cu in SP which was higher. In tilapia fish (*O. niloticus),* the content of Cr, Pb and Zn was higher in the samples collected from HP. Further, the concentrations of Cd, Cu, Pb and Zn detected in fish samples of this study were below the threshold values suggested by FAO and Malaysian Food Regulations (MFR). Nevertheless, the level of Cr was much higher than the recommended limit of FAO for fish consumption. Accordingly, people who could have access to fishing from these ponds are not encouraged to do so where consuming fish with high levels of Cr can cause an adverse effect on their health. However, the current study emphasizes that, in such aquatic ecosystems, fish is considered as an important bioindicator of heavy metal contamination. On the other hand, the SMART ponds have the ability of developing the stormwater runoff quality coming from urban areas. In addition to serving flood control, these ponds can retain sediment and other pollutants associated with settleable particulates.

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Propagation of an Endangered Gymnosperm Tree Species (*Podocarpus neriifolius* D. Don.) by Stem Cuttings in Non-mist Propagator

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ABSTRACT

Podocarpus neriifolius D. Don. (Podocarpaceae), an endangered and the only indigenous gymnosperm tree species, grows naturally in Bangladesh. Seed-based propagation of this species is challenging owing to its inadequate number of mother trees and irregular seed-setting attribute from among a few trees scattered throughout the country. This study weighs the significance and multiplication potentials of this species through rejuvenated stem cuttings with or without the application of Indole Butyric Acid (IBA). The rooting ability of the cuttings was evaluated by treating the cutting bases with 0%, 0.2%, 0.4% and 0.8% (w/v) IBA solution prior to place them in a low cost, non-mist propagation system. Steckling performances of the rooted cuttings were evaluated in the nursery conditions. The study found that the species was amenable to rooting with IBA treatments. The highest rooting percentage (61.3 ± 3.3 %; n = 90) and number of roots per cutting (9.8 ± 1.32; n = 90) were obtained in the 0.8% IBA treatment; however, the longest root and shoot, as well as the highest initial growth performances were obtained in 0.4% IBA treatment. Therefore, rejuvenated stem cuttings treated with 0.4% or 0.8% IBA solution in a non-mist propagator could potentially be an effective method for the clonal propagation of these tree species.

Keywords: Clonal propagation, endangered plant, IBA treatment, rooting ability, stem cutting

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INTRODUCTION

The Podocarps are considered one of the ancient plant groups on earth. Broad-leaved Podocarps, as identified in the Gondwanan fossil, used to be existing 144 million years back (Hill, 1994). Due to subannual light, darkness, and relentless cold weather conditions over extended period of year, broad-leaved foliage of this plant group in high altitude needs to be tough and versatile to endure such weather conditions (Mellick, 2012). Podocarps survived in the long term changing climate with certain physiological modifications including thick, hard, shiny, waxy, and leathery leaves, slow morphological adaptation to evolutionary senescence, restriction to mesic communities, and broad distribution.

Podocarpus neriifolius, Gymnosperm tree species, is one of the 110 species belonging to the genus *Podocarpus* under Podocarpaceae family. The family represents 173 species under 18 genera (Hill, 1994; Quinn & Price, 2003) among a total of 300,000 species of flowering plants including 630 species of conifer in the world (Hill, 1994). Podocarpaceae family was endemic to the ancient super continent of Gondwana and is a classic member of Antarctic floral community (Quinn & Price, 2003). Except for some taller individuals, the species usually grows up to 10-15 m in height and 100 cm in diameter at breast height (dbh) with a clear round bole. The species often bears a dome-shaped crown with whorled branches, brown thin bark, linear or lanceolate leaves of 10-20 cm in length (Figure 1A). While the male flowers of this dioecious tree species are cylindrical catkins with winged pollen (Sacci) grains (Figure 1B), the female flowers are with 2-4 scales (Figure 1C). Receptacles are

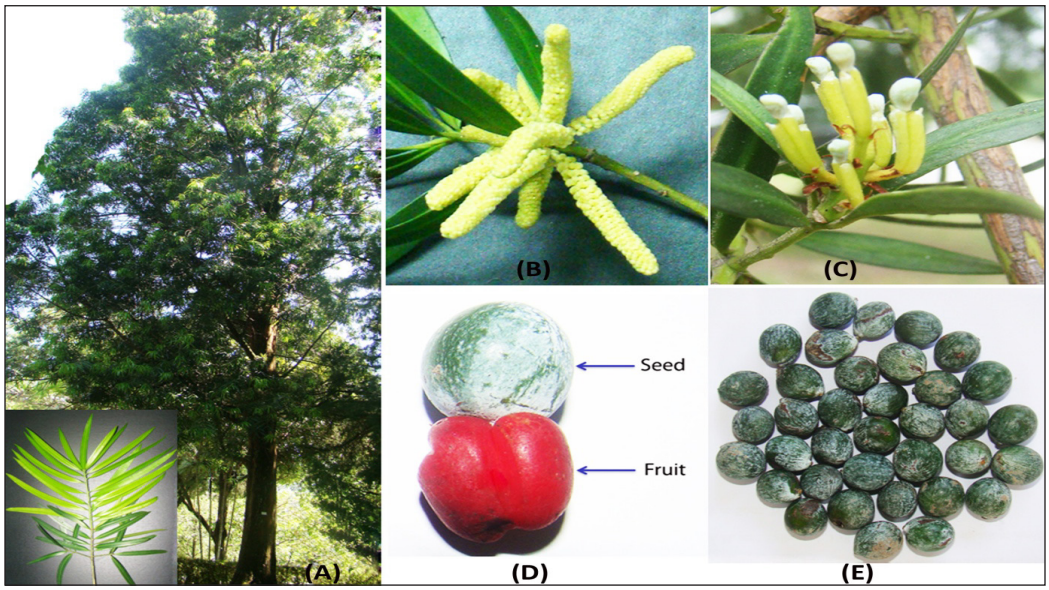


Figure 1. A full grown tree with leaves (inset) (A), male flowers (B), female flowers (C), seed attached with fruit (D) and separated seeds (E) of *P. neriifolius*

thick, fleshy, berry-like swollen, bright red or purple (Figure 1D), edible, and usually eaten by birds. The dark green ovate seeds are 2-3 cm long and 1-2 cm wide (Figure 1E) often with shiny black fleshy parts on ripening. These are also recalcitrant and quickly loss the viability. The species grows in tropical and subtropical closed forests of the eastern Asia and Australia. More specifically, the species is distributed in Nepal, India, Thailand, Vietnam, Malaysia, Indonesia, Philippines, China, Myanmar, Laos, Cambodia, New Guinea, Solomon, and Fiji Islands. In Bangladesh, it is the only conifer species that grows naturally in the forests of Chittagong, Chittagong Hill Tract, Cox's Bazar and Sylhet districts (Bhuiyan et al., 2009). The plant is commonly known as yellow wood, brown wood, brown or black pine, and in Bangladesh, it is called *Banshpata* (Bhuiyan et al., 2009).

Wood of the species is light in weight (Specific gravity 0.46-0.47) and easy to work with (Forest Inventory and Planning Institute [FIPI], 1996). It is also known as pencil wood and being used for making high quality pencils, photo frames, curving, wooden scales, cabinet works, and showpiece frames. The wood yields good quality pulp and can also be used as boxwood. The fruit is edible raw or can be cooked into jam (Bhuiyan et al., 2009). Besides, the species is planted for ornamental purposes around homes and avenues. However, this valuable tree species is now critically endangered in Bangladesh (Anon, 2009) with only 105 trees so far identified in the forests of Chittagong, Chittagong Hill Tracts, and different botanical gardens of the country (Anon, 2009). While some of the trees are over matured with no more reproductive growth, many are immature or unable to produce seed regularly. Due to clear felling, indiscriminate conversion of forest lands to housing, agriculture, transportation, industrialization and lack of mass awareness about the ecological and historical importance of the species, it became endangered and is likely to on the brink of extinction if conservation and much needed propagation measures are not taken immediately. However, it is difficult to propagate the species largely through seed germination due to scarce availability of seed trees in natural forests (Mannan et al., 2001). Only few scattered trees are growing throughout the country and the male and female trees are too far apart to produce profuse seeds. Vegetative propagation is therefore the only alternative to save the species from potential threats of extinction. Among the vegetative propagation means, stem cutting and tissue culture are the most widely used methods for mass clonal propagation for large-scale plantation programs. However, tissue culture involves large-scale investment in initial set up and sub-sequent operational activities, and also requires uninterrupted supply of electricity, which appears to be challenging in many developing countries like Bangladesh. The propagation technique through stem cutting in non-mist propagator (Kamaluddin, 1996) could be a viable solution to this problem. Stem cutting is a severed twig whose base is placed in a moist rooting media to

develop adventitious roots. The method is simple, cost-effective and can be operated in small spaces, anywhere even inside the forest or remote areas without the supply of electricity. The method when coupled with selection of plus trees can result in maximum genetic gain both in yield and quality. It allows continuous production and supply of high quality uniform planting materials with desirable characteristics (Hossain et al., 2014) for large-scale afforestation and reforestation programs (Kamaluddin et al., 1996).

Root development in the cuttings of many tree species is often difficult. Therefore, various rooting hormones are used to enhance rooting ability of desired cuttings. Rooting response can substantially be intensified by applying exogenous rooting hormone IBA, which has already been proved in *Artocarpus heterophyllus* (Hossain et al., 2002), *Pinus caribaea* (Henrique et al., 2006), *Stereospermum suaveolens* (Baul et al., 2009), *Flacourtia jangomas* (Hossain et al., 2011), *Anisoptera scaphula* (Hossain et al., 2014) and *Vitellaria paradoxa* (Akakpo et al., 2014). IBA is more effective in rooting response of cuttings than the NAA and IAA (Henrique et al., 2006; Husen & Pal, 2007; Shen et al., 2010). However, there is a great dearth of published information on the clonal propagation efforts of *P. neriifolius*. That being said, this study was designed to investigate the multiplication potentials of *P. neriifolius* through rejuvenated stem cuttings.

MATERIALS AND METHODS

This study was carried out in the nursery of the Institute of Forestry and Environmental Sciences, University of Chittagong, Bangladesh. The nursery is situated at the intersection of 22°30' N latitude and 91°50' E longitude, which enjoys typically tropical climate, characterized by hot humid summer and dry winter. Mean monthly temperature varies from 21.8°C to 29.2°C in summer and 15°C to 26°C in winter. Relative humidity is the lowest (64%) in February and highest (95%) in June through September. Annual rainfall in the area is about 3000 mm that mostly takes place between June and September.

Stock Plant Management and Shoot Production

Cuttings were collected from the hedge orchard established with vegetative propagules from a 20-year old *P. neriifolius* tree in the nursery. Branches of the stock plants in the hedge orchard were trimmed at the beginning of the study in March, for shoot production. The sprouted juvenile shoots developed on the trimmed branches were collected for cutting preparation in June. Shoots were soaked in water immediately after getting them separated from the stock plants and brought to the laboratory for further processing.

Preparation of Cuttings and Setting in the Propagator for Rooting

Two to four nodal cuttings with two leaves trimmed to one third were used for rooting trials. Cutting length (5.8 - 6.6 cm) and

diameter (3.5 - 3.7 mm) were kept indifferent to avoid possible non-treatment variation among the treatments (Table 1). The cuttings were then briefly treated with fungicide, Diathane M45 (Rohm & Co. Ltd., France; 2g L^{-1} of water), to avoid potential fungal infection and kept in shade for 10 minutes. Effects of exogenous rooting hormone IBA on the rooting ability of the cuttings were explored by treating the cuttings with 0%, 0.2% (2000 ppm), 0.4% (4000 ppm) and 0.8% (8000 ppm) (w/v) IBA solutions. Cutting bases were dipped into the IBA solution for one minute and planted into rooting media (perforated plastic trays filled with coarse sand mixed with fine gravel at a ratio of 4:1) and finally placed into a non-mist propagator for rooting. The trays with cuttings were arranged in the propagator following randomized complete block design.

In the design, 30 cuttings were assigned to each of the four treatments (0%, 0.2%, 0.4% and 0.8% IBA solution). Each block was replicated thrice making a total of 360 cuttings in the experiment. Cuttings of each treatment (90 cuttings) were then planted in 9 trays, 3 trays (10 cuttings each) for each replication. The cuttings were watered once only just after setting them in the propagator. A light water spray was done every day early in the morning (before 7 am) and late afternoon (after 6 pm) until transferring the rooted cuttings from the propagator.

Propagator Environment

Relative humidity of around 85-95% was maintained in the propagator. Propagator was kept open for a short period of time once in the morning (before 7:0 am) and once in the afternoon (after 6:0 pm) every day for facilitating gas exchange. A bamboo shed lined with jute mat was placed over the propagator to avoid excessive solar heat. Thus, the photosynthetic photon flux measured with quantum sensors (SKP 215, Skye Instruments Ltd., UK) and data logger (Datahog2, SDL5360, Skye Instruments Ltd., UK) inside the propagator was reduced to 12% of the full sun. During the rooting experiments, mean maximum and minimum temperature ranged between 31°C and 23°C.

(A) (B)

Figure 2. Non-moist propagator (A) and the cuttings in rooting media (B) inside the propagator

Transferring the Rooted Cuttings to Polybags

The cuttings rooted after 14 weeks of setting them in rooting media in propagator. The rooted cuttings were weaned (hardened) before transferring them to polybags by keeping the propagator open at night for three subsequent days and at day and night for another three days. After weaning, all the rooted cuttings were transferred into polybags filled with forest soil mixed with decomposed cow dung at a ratio of 3:1. Before planting into the polybags, rooted cuttings were assessed for length and diameter, root and shoot number and the length of longest root and shoot developed in each cutting. After transferring the rooted cuttings into the polybags, they were kept under shade for one week and then placed under sun for growing. One year after transferring the rooted cuttings into the polybags, survival percentage and total height of each rooted cutting were measured for their growth assessment.

Data Recording and Statistical Analysis

Mean growth variations attributed to treatments were evaluated using the analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT). Rooting percentages were adjusted accordingly using arcsine transformation formula before placing the data into analysis of variance (Islam et al., 2011). Rooting percentages, number and length of roots and shoots developed for control and the treatments were compared at $p \leq 0.05$ (ANOVA and DMRT).

RESULTS

Rooting Ability of *Podocarpus neriifolius* Cuttings

Cuttings of *P. neriifolius* started rooting from the 10th week and completed in the 14th week in propagator (Figure 2). However, shoot development started from the 12th week and continued until 15th week. Rooting performance of the stem cuttings was assessed after 15 weeks of setting the cuttings for rooting trial in the non-mist propagator. Percentage of cuttings rooted ranged from 30 to 61 among the treatments. IBA was found to significantly enhance the rooting in the cuttings. The highest rooting percentage (61%) was recorded when cuttings were treated with 0.8% IBA solution followed by 57% with 0.4% IBA and the lowest (30%) in non-treated cuttings (Figure 3). The rooting percentage in 0.4% IBA treatment did not significantly vary from that in 0.8% IBA treatment.

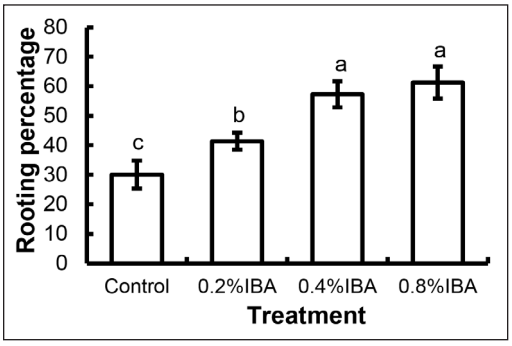


Figure 3. Percentage of *P. neriifolius* cuttings rooted in different IBA concentrations 15 weeks after setting them in propagator for rooting

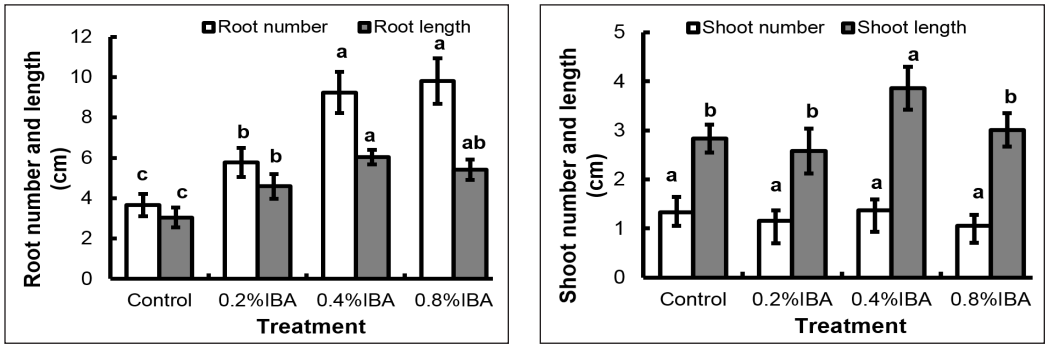
Root Number and Length

Average number of roots produced in each cutting varied from 3.7 to 9.8 among the treatments. The maximum number of roots

(9.8) was produced in cuttings treated with 0.8% IBA solution followed by 9.2 in 0.4% and the lowest (3.7) in the control. IBA application remarkably enhanced the number of roots produced per cutting. However, the number of roots was not significantly different in the cuttings treated with 0.4% and 0.8% IBA solutions (Figure 4A and Figure 5). Like rooting percentage and root number, length of the longest root for cuttings was also significantly affected by IBA treatment. The longest root (6.0 cm) was recorded in the cuttings treated with 0.4% IBA solution followed by 5.4 cm in 0.8%, 4.6 cm in 0.2% and the shortest 1.4 cm in the control.

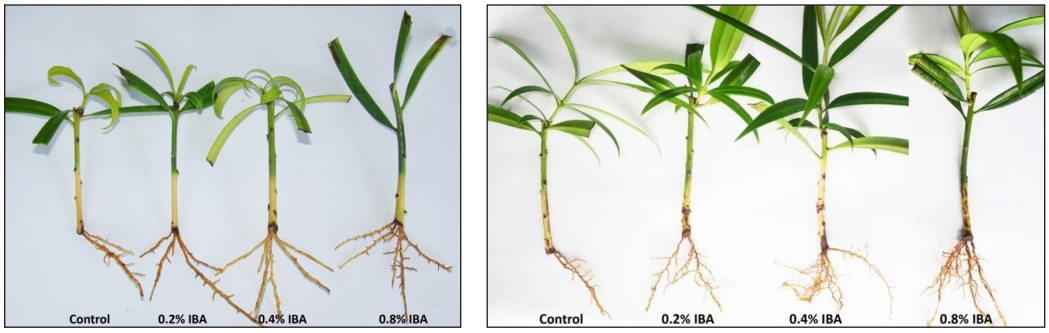
Number of Shoot and Shoot Length

Although there was no significant difference in the number of shoot developed in cuttings, shoot length remarkably increased when treated with IBA solutions. Average number of shoot and shoot length produced per cutting ranged from 1.0 to 1.4 and 2.6 cm to 3.8 cm among the treatments, respectively. Shoot length was significantly higher in the cuttings treated with 0.4% IBA solution than in other treatments (Figure 4B). However, there was no meaningful variation in shoot length developed in the cuttings in other treatments.



(A) (B)

Figure 4. Development of roots (A) and shoots (B) in the cuttings 15 weeks after planting in the propagator



(A) (B)

Figure 5. Rooting in cuttings treated with different concentrations of IBA solution 12 weeks (A) and development of shoots 15 weeks (B) after setting the cuttings in the non-mist propagator

Cutting Morphology

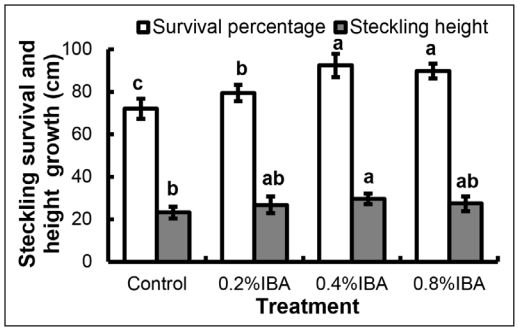
The average length and diameter of the cuttings varied from 5.5 cm to 6.1 cm and 3.5 mm to 3.7 mm, respectively, among the cutting types. There was no significant difference in the mean cutting length and mean diameter among the treatments at $p \leq 0.05$ (ANOVA and DMRT) (Table1). Therefore, there was no remarkable effect of cutting length and diameter on rooting percentage, root number, root length, shoot number and shoot length in the cuttings. Actually, the cutting length and diameter were kept indifferent in various treatments purposively to avoid non-treatment variation among the cuttings.

Table 1
Mean length and diameter (±SE of mean) *of cuttings rooted under different concentrations of IBA solution. The same superscript letter(s) indicates no significant difference at p≤0.05 level (ANOVA and DMRT)*

Variables	Treatments			
	Control	0.2% IBA	0.4% IBA	0.8% IBA
Mean cutting length (cm)	5.76^{a}±0.17	5.5^{a}±0.62	6.1^{a}±0.29	5.9^{a}±0.31
Mean cutting diameter (mm)	3.73^{a} ± 0.25	3.6^{a} ± 0.17	3.59^{a} ± 0.25	3.71^{a} ± 0.26

Steckling Growth in the Nursery Condition

The survival percentage of *P. neriifolius* stecklings (rooted cuttings) was also pointedly enhanced when treated with IBA solutions. Almost 72% to 93% stecklings were thriving among the treatments in the nursery one year after transferring them into the polybags (Figure 6). The highest survival percentage was recorded in the stecklings developed with 0.4% IBA treatment followed by 0.8% IBA and the lowest (72%) was in the control (Figure 6). Gradual increment of survival percentage was also noticed with increasing concentration of IBA up to 0.4% IBA solution. The average height in the stecklings was also the maximum (29.6 cm) in the cuttings developed with 0.4% IBA solution followed by 0.8% or 0.2% IBA and lowest (23.1 cm) was in the control. The survival percentage and initial growth performance of the out-planted stecklings were satisfactory (over 90%) one year after transplanting in the field. However, due to insufficient number of plantable stecklings from the treatment with 0.2% IBA and control, stecklings treated with 0.4% and 0.8% IBA were only planted for observation in the field. No significant variation was observed in steckling survival percentage and height growth between these two treatments.



(A)

(B)

Figure 6. Survival percentage and steckling height of *P. neriifolius* cutlings developed in different concentration of IBA treatments one year after transferring in the polybags

DISCUSSION

Rooting Ability of *Podocarpus neriifolius* Cuttings

Podocarpus neriifolius D. Don. is an endangered and the only naturally grown indigenous gymnosperm tree species in Bangladesh (Mannan et al., 2001). Due to the scarcity of mother trees or scattered dioeciuos productive trees with irregular seeding habit, regeneration of the species by seed germination is not practical. Initiatives have been taken for mass propagation of this endangered tree species through rejuvenated stem cuttings with IBA treatment in low-cost non-mist propagation system. From our study, the species was found amenable for vegetative propagation through stem cutting with or without rooting hormone. However, rooting ability of the cuttings was greatly influenced with IBA treatments. The maximum rooting percentage (61) was recorded in the cuttings treated with 0.8% IBA followed by 0.4% IBA and the minimum (30) was observed in the cuttings without any treatment (control). Formation of roots is a complex process and a vital step for plant propagation through stem cutting for many important species (Pop et al., 2011). Among the rooting hormones IBA was considered as the most effective in rooting in the cuttings. Henrique et al. (2006) reported that *Pinus caribaea* var. hondurensis cuttings treated with IBA produced higher percentage of rooted cuttings than those treated with NAA. Husen and Pal (2007) reported that overall rooting response was better in the treatment with IBA rather than with NAA in *Tectona grandis* stem cuttings. Shen et al. (2010) recorded the highest rooting percentage (68%) on medium supplemented with 5 mM IBA where IAA and NAA failed to root. Rooting percentage of cuttings has been reported to influence with application of exogenous rooting hormone IBA. Therefore only IBA was tested to intensify the root formation in *P. neriifolius* cuttings in this study. Applied auxin (IBA solution) remarkably increased the rooting percentage in cuttings of both conifer and broad leaved tree species including *Pausinystalia johimbe* (Tchoundjeu et al., 2004), *Baccaurea sapida*

(Abdullah et al., 2005), *Sloanea suaveolens* (Baul et al., 2009), *Flacourtia jangomas* (Hossain et al., 2011), *Anisoptera scaphula* (Hossain et al., 2014) and *Velleia paradoxa* (Akakpo et al., 2014). In this study, we noticed the highest rooting percentage (61) in the cuttings treated with 0.8% IBA solution, which was significantly higher in the cuttings treated with 0.2% IBA or in control. Similar results were reported by Hossain et al. (2014) and mentioned that maximum rooting percentage was obtained in *A. scaphula* cuttings with 0.8% IBA treatment. However, other researchers noticed significantly higher rooting percentage with 0.4% IBA solution. For examples, Hossain et al. (2002, 2004) reported significantly enhanced rooting percentage in the cuttings of *Artocarpus heterophyllus*, *Swietenia macrophylla* and *Chukrasia velutina*, respectively with 0.4% IBA solution. Tchoundjeu et al. (2004) obtained better rooting percentage with 0.4% IBA treatment in *P. johimbe*, and Abdullah et al. (2005) in *B. sapida* cuttings. Again, Baul et al. (2009) stated better rooting performance in *S. suaveolens* cuttings with 0.4% IBA solution. Neghas (2002) noticed suggestively decreased rooting percentage and root number in *Juniperus procera* when they were treated with more than 0.4% IBA concentration. Actually the required concentration of exogenous IBA for rooting varied based on species, nature (woody or soft cuttings) and state of the cuttings. The doses ranged from 0.1% (Baul et al., 2011) to 10.0% (Lee & Bilderback, 1990). In the present study we noticed that 0.8% IBA solution was suitable for rooting of *P. neriifolius* in 10 to 14 weeks, seemingly a hard-to-root species.

Root number per cutting was also significantly enhanced with the treatments of exogenous rooting hormone IBA. Maximum number of root per cutting (9.8) was achieved with 0.8% IBA solution followed by 9.2 with 0.4% and lowest number was exhibited in the cuttings without IBA treatment. Similar results were reported by Hossain et al. (2014). They mentioned that the highest number of root was produced in *A. scaphula* stem cuttings treated with 0.8% IBA. Akakpo et al. (2014) explored maximum number of root (11) in *V. paradoxa* cuttings with 5000 ppm (equivalent to 0.5%) IBA solution. Number of root produced per cutting in different species with various concentrations of IBA was reported by several authors. Hossain et al. (2002) reported significant increment of root number when cutting bases were dibbed in 0.4% IBA solution. Kamaluddin and Ali (1996) reported significantly increased number of roots in *Azadirchta indica* cuttings with IBA treatment. Again Kamaluddin et al. (1998), in a separate experiment, noticed that significantly enhanced rooting ability of *C. velutina* cuttings by applying exogenous rooting hormone. Besides, Hossain et al. (2004) stated that mean root number of *S. macrophylla* cuttings was suggestively higher in the cuttings treated with rooting hormone IBA. Number of roots per cutting in the 0.8% IBA treatment was almost similar with the cuttings treated with 0.4% IBA without any significant variations.

Like rooting percentage and root number per cutting, root length was also enhanced with the IBA treatment in the cuttings. However, the longest root length (6.0 cm) was recorded in the cuttings treated with 0.4% IBA solution followed by 5.4 cm with 0.8%, 4.6 cm with 0.2% and shortest roots in control cuttings. There are some reports mentioning the enhancement of root length in the cuttings. Hossain et al. (2004, 2011) and Alam et al. (2007) reported significant increase of root length in presence of 0.4% IBA solution. However, Hossain et al. (2014) reported maximum root length (7.7 cm) in 0.8% IBA treated cuttings followed by 0.4% IBA and minimum (2.5) in the control. In fact, the applied IBA indirectly influences the speed of translocation and movement of sugar at the base of cuttings and subsequently accelerates rooting in the cutting base (Haissig, 1974, 1982). Basically, speeding up of root formation in cuttings is considered as an advantage and the earlier the development of roots, the greater the chances for survive and thrive later on.

Shoot Number and Length

There was no significant difference in the number of shoot developed in cuttings. However, shoot length was remarkably increased with IBA application. Shoot length was significantly higher in the cuttings treated with 0.4% IBA solution than that in other treatments (Figure 4B). However, there was no relevant report found that explained the variation in shoot length of the cuttings under different treatments. The maximum shoot length was recorded in the cuttings treated with 0.4% IBA which might be due to the optimum energy partitioning for the production of root and shoot with the help of 0.4% IBA solution. Whereas the cuttings with low concentration (0.2%) or without IBA treatment did not get sufficient amount of rooting hormone to develop the root or shoot in the similar fashion of 0.4% IBA treatment. On the other hand, the cuttings treated with comparatively higher concentration (0.8%) of IBA produced more roots than the shoots. This indicates that the rooting hormone influenced the cuttings for allocating more energy for root than the shoot development.

Steckling Capacity of *P. neriifolius* Cutlings

Survival percentage and initial growth performance of the cutlings (rooted cuttings) of *P. neriifolius* was greatly influenced when rooted with IBA treatment. The highest survival percentage (93) was in the cutlings rooted with 0.4% IBA solution followed by 85% with 0.8% IBA and lowest (73) from the control in nursery condition one year after transferring them into the polybags. Likewise, the maximum height growth was observed in the same treatment with lowest in control. The average steckling height was also maximum (29.6 cm) in the cuttings rooted with 0.4% IBA followed by 0.8% or 0.2% IBA and the lowest (23.1 cm) in the controlled cuttings. Similar outcomes were reported by Hossain et al. (2011) for *F. jangomas* cutlings and mentioned that the highest survival and

initial growth performance were found in the cutlings rooted with 0.4% IBA solution. In a separate study Hossain et al. (2014) also mentioned higher survival percentage (83.3%) and initial growth of *A. scaphula* cutlings developed with 0.8% IBA solution compared to the other treatments including control which is unidirectional with the findings of Nath and Barooah (1992). However, the factors affecting the survival potentials of the stecklings rooted with IBA were not possible to clarify due to the lack of related references. Higher number of root in the cuttings developed with 0.8% or 0.4% IBA solution might have contribution in better survival and initial growth performance of the stecklings in the nursery and field condition.

CONCLUSION

Podocarpus neriifolius is the critically endangered and the only naturally growing gymnosperm in Bangladesh that needs additional management treatments for its survival in the natural stands. In this study, efforts have been made for mass clonal propagation of the species through stem cutting for large-scale plantation programs. The results revealed that the species is hard-to-root but amenable to rooting with rooting hormone. Cuttings treated with 0.4% to 0.8% IBA solution can significantly enhance the rooting ability, survival potential, and initial growth performance in the nursery and in the field. Rooting percentage and root number per cutting were maximum in the 0.8% IBA treated cuttings, but were not significantly higher than those in the 0.4% IBA treatment. Moreover, average shoot length, survival percentage and initial growth performance of the stecklings in nursery condition were significantly higher in the rooted cuttings developed with 0.4% IBA treatment. Survival potentials of the out-planted cutlings developed through 0.4% or 0.8% IBA treatment were also satisfactory one year after transplanting. Therefore, it can be concluded that the species could successfully be propagated from rejuvenated stem cuttings with 0.4% or 0.8% IBA treatment in the low-cost, non-mist propagator for mass clonal propagation and plantation programs.

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Food Items and Foraging Sites of the Oriental Pied-Hornbill (*Anthracoceros albirostris*) during Breeding Season in Sungai Panjang, Sabak Bernam, Malaysia

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ABSTRACT

The Oriental pied-hornbill (*Anthracoceros albirostris*) is highly adaptable to habitat changes compared with other hornbill species. Although the species is omnivorous, their diet varies between seasons and can be restricted by food availability and abundance in the forest. Recently, Oriental pied-hornbills were spotted breeding at a human settlement in the rural area of Sungai Panjang, Selangor in abandoned clay jars. As the female seals itself in the nest, the male plays an important role in provisioning its partner and the chicks. Therefore, this study aimed at understanding the male's food items and foraging sites selection in Sungai Panjang during the breeding season. Three hornbill pairs were monitored between 2009 and 2011 to examine their foraging activities. Video recorders were used and the males were followed every alternate day to their respective foraging sites. Results showed the number of visits made and foods brought back by the males to the nests were dependent on the location of their nests. The average visits recorded were between four and 12 times a day (mean visit per day: 9.07 ± 3.40). In addition, they utilised different foraging areas, such as oil palm plantations, orchards and forest patches. Interestingly, 50% of animals were brought back to the nests after visits to plantations compared with fruits that were gathered mainly from the orchard (48%), while the rest (2%) was unidentified. Forest patches were the least utilised site. In sum, the species has taken advantage of Sungai Panjang's agricultural background particularly during its breeding season. The findings confirm the species high adaptability to disturbed habitat.

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Keywords: Adaptability, agricultural land, diet, foraging activity, Oriental pied-hornbill

INTRODUCTION

The Oriental pied-hornbill (*Anthracoceros albirostris*) is common in Asia (Del Hoyo et al., 2001). However, in some areas like the Thai-Malay Peninsula, Laos and Vietnam, the population is threatened due to indiscriminate deforestation (Poonswad, 1995; Vyas, 2002), hunting by local tribes (Sethi & Howe, 2009) as well as fledgling trading (Wells, 1999). Despite these setbacks, the species is known to have high adaptability to habitat changes, provided ample supply of food and large mature trees for nesting are present (Chong, 1998). The species can also be found foraging and nesting in logged forest (Datta & Rawat, 2004). This is likely due to the species having broad diet in exploiting food resources compared with other hornbills (Kitamura et al., 2009). Therefore, the species is listed as 'Least Concern' under the IUCN Red List of Threatened Species (BirdLife International, 2016). Its subspecies, the Southern pied-hornbill (*Anthracoceros albirostris convexus*) is common in the southern part of Peninsular Malaysia and can be differentiated by the white outer-tail feathers (Robson, 2002). According to Ismail et al. (2015), this subspecies has been increasingly observed foraging in the agriculture area of Sungai Panjang, Sabak Bernam, Malaysia. However, there have been no detailed studies to date on their food items and foraging sites selection in such area.

Generally, the hornbill's diet may vary between seasons depending on food availability and abundance. Having broad habitat preferences, the Oriental pied-hornbill in particular can thrive very well in modified landscapes. Many studies have highlighted the hornbill's foraging activities in undisturbed or semi-disturbed areas including logged forest (Datta & Rawat, 2003, 2004). On the contrary, the hornbill's foraging activity in agricultural lands has never been reported, unlike those in the logged forest (Sethi & Howe, 2009) and human habitation (Chong, 1998). This is probably due to the infrequent or rare sightings of the species in these areas. Ismail et al. (2015) reported that several groups of Oriental pied-hornbills (subspecies: Southern pied-hornbill) in Sungai Panjang, Sabak Bernam have recently adapted to an unconventional method of nesting by using abandoned clay jars in the area. Thus, there is a high possibility that the population is depending on the extensive agricultural land for food to sustain themselves during the breeding period. Like most hornbills, the Oriental pied-hornbills are monogamous and often establish relationship over a long period of time (Kemp, 1995; Ng et al., 2011). Hence, strong bonding develops between couples and best displayed particularly during the breeding activity. Ismail et al. (2015) reported that as the females were sealed inside clay jars, the males played an important role to provide for their partners and the chicks. Thus, this study examines

the food items and foraging sites selection of the Oriental pied-hornbills (subspecies: Southern pied-hornbill), particularly during their breeding seasons.

METHODS

Study Area

Sungai Panjang (N 3° 44', E 101° 5') is located in the Sabak Bernam District of Selangor (Figure 1). Palm oil plantations and paddy fields are the main agriculture activities here. Most of the lands were converted to support this industry. Sungai Karang Forest Reserve (SKFR) is the closest natural and undisturbed habitat, approximately five kilometres from the study area. With an area of more than 50,000 ha, it is home to at least five different hornbill species, including the Oriental-pied hornbill (Ismail et al., 2015). However, as the number of nesting hornbills began to increase in Sungai Panjang, this study was conducted to investigate their foraging activity in this particular area.

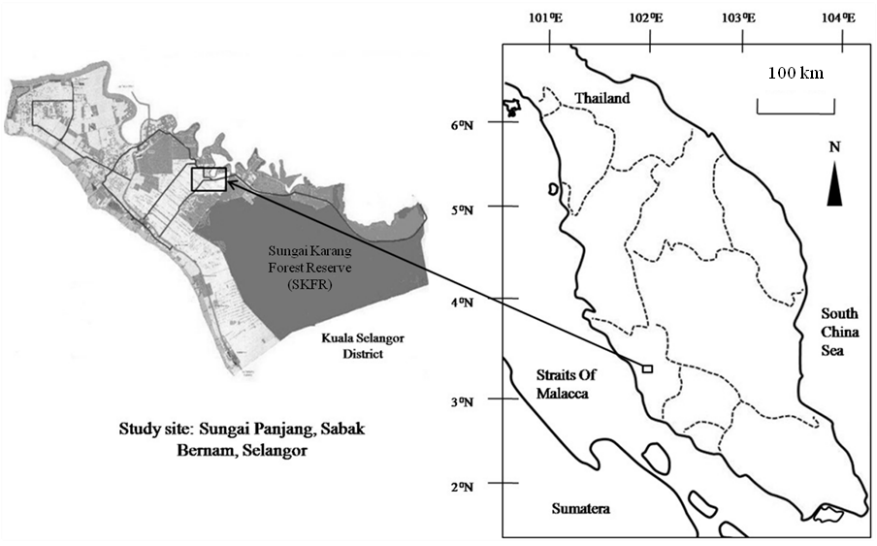

Figure 1. Map of the study area, Sungai Panjang, Sabak Bernam, Selangor

Sites Selection and Surveys

Three hornbill pairs were monitored during their breeding activities at three different sites between 2009 and 2011. Table 1 shows the description of the study sites. Each of the breeding sites was located close to a villager's house and in an abandoned clay jar on the ground. These jars were 58 cm in length, 39 cm in width, with an opening diameter of 12 cm. Ismail et al. (2015) had reported on this unique nesting selection. The Oriental pied-hornbills usually begin to nest in early January (sometimes earlier) and it could last until late April, lasting for 70-78 days. To study the males' provisioning activity, three video recorders (Sony HDR series), were plugged into the main electricity from the nearby

residential areas for continuous recording. The recorders were set up at a distance between three to five metres from the nests. Most of the diets supplied by the males were identified from these recordings. In order to study the males foraging activity, they were followed every alternate day, during the breeding period, by foot or vehicle (if accessible). The males were individually identified by the unique markings on their casques and features. However, most of the data for this particular study was collected towards the end of the 2011 breeding season. More efforts were focused on surveying the different habitats available as well as identifying the actual habitat in which the males foraged on as they usually made short stops before they proceeded to the foraging sites. The presence of other non-breeding hornbill individuals also made the identification process difficult earlier but this was overcome later on as the researchers became more familiar with the subjects. Foraging distances varied between nesting locations and can range from 10 metres and up to one kilometre. Field observations were aided by binocular (Nikon II Egret). Nest inspection was only done if deemed necessary and only after the male had left so as to not disturb the provisioning activity.

Table 1

Description of the study sites in Sungai Panjang, Sabak Bernam

Site	Longitude/ Latitude	Site Description
1	3°43'30.90" N 101°04'43.36" E	Clay jar abandoned near a villager's house and surrounded by forest patches and palm oil plantation
2	3°43'46.03" N 101°05'48.60" E	Clay jar abandoned in an orchard and close to a palm oil plantation
3	3°43'58.60" N 101°05'57.10" E	Clay jar abandoned in an orchard and close to forest patches

Data Analysis

Kruskal-Wallis and Mann-Whitney U tests were employed to test the differences of the visits made by the males between the nesting sites and foraging habitats. Pearson Chi-Square test was also employed to assess the association between the foraging habitats and the types of food brought back by the males. All statistical analysis was done using Statistical Package for the Social Science (SPSS). Comparisons were made at 95% level of significance.

RESULTS

The hornbills' activity and behaviour were monitored for more than 1700 hours. In general, the frequency of the males' visits to the nests varied, between four and 12 times a day (mean visit per day: 9.07 ± 3.40) with increasing pattern of visits

recorded as the times progressed. Figure 2 shows the variations in the visits made by the males at each nesting site. In terms of yearly visits, no significant differences were recorded in Site I (S1: X^2 (2) = 3.99, p = 0.136, N = 720). However, nest in Site II recorded significantly lower feeding visits in 2010 compared with 2009 and 2011 (X^2 (2) = 9.45, p = 0.009, with mean visit per day of 11.13 ± 1.38 for 2009, 10.7 ± 1.4 for 2010 and 11.24 ± 1.4 for 2011). This coincides with the sudden increase in the number of Long-tailed macaques (*Macaca fascicularis*) in Site II in 2010, causing the male to spend more time guarding its nest. The videos also recorded the birds, clearing the debris and food waste near their three nests between 0700 to 1900 hours. Such behaviour suggests that the males play an active role in protecting the nests from potential predators in the area. As for the nest in Site III, significant increase in terms of visit frequency in 2010 (U = 645.5, p = 0.002, with mean visit per day of 10.64 ± 1.5 for 2010 and 11.62 ± 1.3 for 2011) were recorded. Prolonged stays or exposure of the pairs particularly the males to the new environment could have played a role. The 2009 data for Site III was not included as the breeding attempt was from a different pair which was not successful. Interestingly, lower male visits (total) were recorded at Site I (4.40 ± 0.79 visit per day, N= 720) as compared with Site II (11.02 ± 1.40 visit per day, N = 1980) and Site III (11.13 ± 1.48 visit per day, N = 1320). Proximity of the nest to the foraging areas could have contributed to the differences. In terms of utilisation of foraging area, the males studied relied upon different habitats available including plantation, orchard and forest patch for food sources. Highest visits by the males were recorded in plantations (42% out of the total number of visits) followed by orchards (40%) and forest patches (18%) (Figure 3).

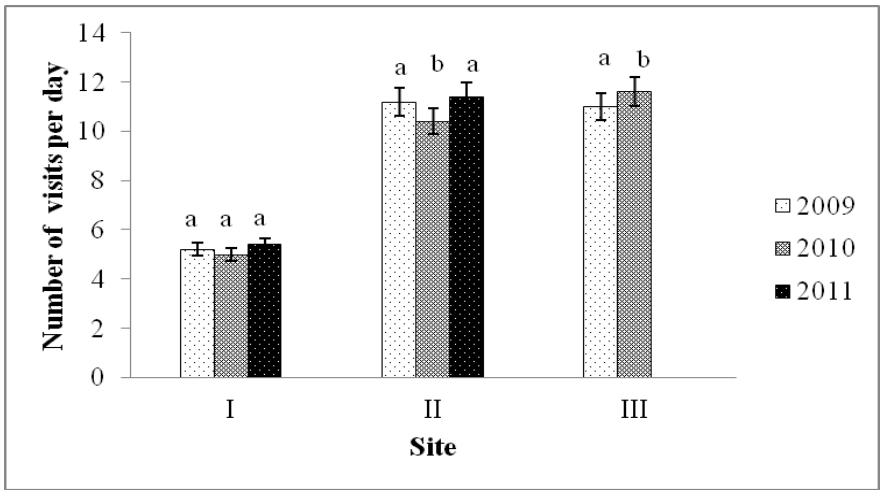

Figure 2. Visits made by the males at the different nesting sites throughout the study period (N = 4020) * 'a' and 'b' are statistically different at p = 0.05

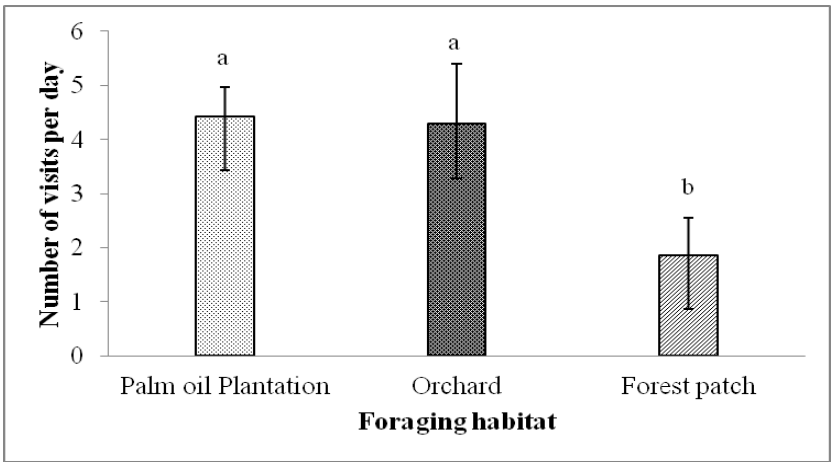

Figure 3. Types of habitat visited by the male Oriental pied-hornbills during the study (N = 420) * 'a' and 'b' are statistically different at $p = 0.05$

In this study, the diet of the Oriental pied-hornbill (subspecies: Southern-pied) consists of fruits such as wild berry (*Vitis difussa*) (6%), palm oil fruit (*Elaeis guineensis* Jacq.) (7%), banana (16%), papaya (16%), cherry (*Muntingia calabura*) (5%), to small animals such as bird's chicks (2%), insects i.e. leaf bugs, grasshoppers, praying mantis (35%), other arthropods (6%), snails (3%) and reptiles (2%). However, the remaining 2% could not be identified. The males supplied their partners with relatively higher proportion of fruits (50%) compared with animals (48%). Details of the hornbill's diet are shown in Figure 4. Significant association was found between foraging habitats - plantation and orchard - and the types of food brought by the males. In general, small animals and insects were predominantly caught by the

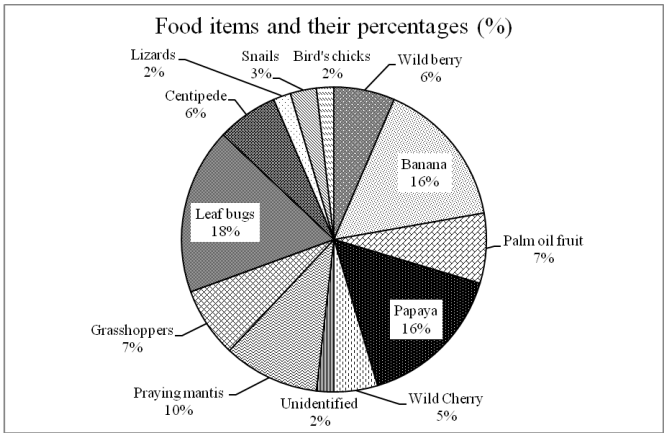

Figure 4. Food items brought back by the males Oriental-pied hornbills in the study

males after their visits to palm oil plantation (χ (33) = 216.12, $P < 0.01$, $N = 115$). On the other hand, fruits (not including palm oil fruit) were mostly obtained from the orchard ($\chi(96)$, $p < 0.01$, $N = 115$). Due to limited visits made to the forest patch, no association was found for the area.

DISCUSSION

Generally, the male fed its partner for the duration of the nesting or concealment. Once the eggs hatched, the number of visits to the nest also increased, corresponding to the higher demand for food supply by the nestlings. The male also protected the nest and its content by removing excess foods outside the nest's opening. Such behaviour is viewed as important to avoid unwanted attraction from potential predators and is highly unique as the conventional tree-nesting does not require such interaction due to its height (Ismail et al., 2015). Throughout the study period, the food items were mainly gathered from plantation and orchard. Nest sites located near orchards and plantation (Site II and III) are linked to higher number of visits or returned trips made by the males. The selection of nesting location may have been influenced by the presence of food sources (Kemp, 1995; Mudappa & Kannan, 1997). Prolonged utilisation of the foraging sites following consecutive breeding activities in the same site may also be a contributing factor. Palm oil plantation and orchard remained as areas heavily visited by the males - suggesting they provided very important food sources (including insect and fruits). However, this particular result could be biased as researchers would have lost sight of the males as they moved swiftly between areas. Moreover, most of the data recorded was collected towards the end of the 2011 breeding season.

Although all hornbill species are omnivorous, most rely heavily on fruits (Kemp, 1995). Hence, hornbill diets are largely dependent on fruiting seasons which can also be both temporally and spatially restricted. Although figs which are commonly associated with their diet and are available all year round (Poonswad et al., 1998), this study did not record any type of figs being consumed by the birds. This could be because the surrounding areas have been converted either into plantation or orchards, leaving only small area of forest strips or patches. Moreover, it has been reported that the chicks require more protein and the food brought by the male contains more animal components as the nestling progresses (Teo, 2017). However, this was not investigated in detail during this study. In addition, the nature of the agricultural areas in the study area had allowed the birds to forage for foods all year-round. There is a high possibility of the population dispersed to Sungai Panjang due to competition for limited resources. Competition with other species may have also led the hornbill to shift the location of their foraging activity outside their natural habitat, the SKFR. No sighting of the males leaving the Sungai Panjang area was observed. However, further study is needed to ascertain this as the sampling effort is not exhaustive. As most of the land activities in Sungai Panjang

are for agricultural use, the Oriental-pied Hornbill could also play an important role as a seed disperser agent, particularly for the orchards and fruit farms in the area. Hence, the public awareness should be increased to protect the species and their population in the future.

CONCLUSION

The Oriental pied-hornbills in Sungai Panjang have taken advantage of Sungai Panjang's agricultural land, particularly during the breeding season. The findings prove the species high adaptability to disturbed habitat.

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Review Article

Enhancers in Proboscis Monkey: A Primer

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ABSTRACT

Enhancers are indispensable elements in various developmental stages, orchestrating numerous biological processes via the elevation of gene expression with the aid of transcription factors. Enhancer variations have been linked to various onset of genetic diseases, highlighting their equal importance as the coding regions in the genome. Despite the first enhancer, SV40 been discovered four decades ago, the progress in enhancer identification and characterization is still in its infancy. As more genome sequences are made available, especially from that of the non-human primates, we can finally study the enhancer landscape of these primates that differs evolutionarily from that of human. One interesting genome to investigate is that of the proboscis monkey as it is deemed as one of the most ancient primates alive to date with unique morphological and dietary characteristics; it is also one of the IUCN endangered species with the strong demands for immediate conservation. In this review, we provide some justifications and considerations of selecting the proboscis monkey as a model for enhancer landscape discovery. It is hoped that more conservation research and protective measures can come in time to prevent this species from extinction.

Keywords: Conservation, enhancer, gene regulation, primate, proboscis monkey

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INTRODUCTION

Enhancer, as its name suggests, is an essential regulatory DNA element capable of enhancing and elevating gene transcription and all other processes that

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occur at post-transcription as described in the central dogma of molecular biology (Pennacchio et al., 2015). Enhancers are vitally indispensable as it plays major roles in orchestrating evolutionarily important phenotypes as well as biological processes at numerous developmental stages (Pennacchio et al., 2015). The magic the enhancers have that differs them from gene promoters is that they can regulate adjacent and distal genes in the bidirectional orientation and locality-unrestricted manner (Melo et al., 2013; Natoli & Andrau, 2012).

Despite the fact that the first enhancer, SV40 was discovered over four decades ago by Banerji et al. (1981), it was only recently that enhancers were once again being promoted into the limelight of the molecular biology field for its significance in disease-related genetics as the first disease-related enhancer was found in Hirschsprung disease (Grice et al., 2005). Another crucial driving force for this phenomenon is no other than the emergence of next generation sequencing which has unravelled genome sequences of various species (Baker, 2012). The completion of genome sequencing of human and other famous model organisms had revealed more than just previously undiscovered evolutionarily conserved non-coding regions but also functionally conserved (but not necessarily sequence-wise conserved) regions believed to function as enhancers (Melton et al., 2015). On the side note, among the emerging genome sequencing projects initiated in the late 20th century (Gordon et al., 2016; Prüfer et al., 2012; The Marmoset Genome Sequencing and Analysis Consortium, 2014), the non-human primates are one of the major highlights as they represent the closest evolution counterparts to human and the high similarities they share with human in terms of coding and non-coding regions are very valuable for biomedical genetic studies especially (Harding, 2013).

The proboscis monkey, *Nasalis larvatus*, which is endemic to Borneo Island is one of the interesting non-human primate subjects to study. This species is deemed to be the most primitive colobine based on its morphological characteristics as well as exceptional diploid number of 2n=2x=48 (Chiarelli, 1966; Soma et al., 1974; Stanyon et al., 1992). These ancient adaptive traits above that are possessed by none other than the proboscis monkey, are very beneficial for the investigation of the enhancer landscape in primates, especially in its most ancient form to study on how evolutionary divergence of these enhancers would lead to phenotypic variations and speciation. As enhancers are known for their rapid evolution especially across mammals and recently evolved enhancers are found to be the dominators in mammalian regulatory landscapes (Villar et al., 2015), thus it is interesting to discover the effects of gain-of-function or loss-of-function of these enhancers on the emergence of genetic disease throughout the divergence process in primates. The variations in enhancer sequences are also known to be associated with the onset of various developmental and genetic diseases known to date in human (Kim et al., 2011; Kleftogiannis

et al., 2015). Furthermore, this monkey species is currently listed as endangered by IUCN (Meijaard et al., 2008). Therefore, by exploring the enhancer landscape in its most ancient form in the most primitive primate like the proboscis monkey, we can understand how evolution had changed the enhancer landscape in primates and further aid in conservation research like antibody synthesis against elephant endotheliotropic herpesvirus (EEHV) (Kochagul et al., 2018), pathogen combatting in white-nose syndrome in bats (Palmer et al., 2018) as well as the toxicology gene expression studies on endemic *Rasbora* fish (Lim et al., 2018b). Besides, the proboscis monkey enhancer landscape is useful in the understanding of the adaptive phenotypic traits that occur in the environment for displaced wildlife (Luo & Lin, 2016; Vogt, 2017) for more effective conservation measures and strategies in future. In this review, we provide some justifications and considerations of selecting the proboscis monkey as a model for enhancer landscape discovery and conservation.

Gene Regulation

The expression of gene in cell and tissue is governed by DNA components termed the regulatory elements, they control the amount of gene products produced spatially and temporally at different developmental stages (Laybourn, 2001; Scott, 2000). The regulation of gene expression is conducted in various ways and forms ranging from chromatin remodelling, transcription initiation, transcription, transcript modifications, mRNA degeneration, translation initiation, translation, posttranslational modifications to protein transport and protein degradation. Each of these stages are tightly monitored to ensure the survivability and adaptability of the host organism towards diverse environmental stimuli (Laybourn, 2001; Scott, 2000). In eukaryotic organisms, the gene regulation mechanism is much more complex as compared to their prokaryote counterparts as it involves multifaceted networks and numerous cross-acting regulatory elements (Scott, 2000; Watson et al., 2014).

The regulatory modules such as the promoter and enhancers determine the expression level of the gene via transcription factor binding. The core promoter is present in all eukaryotic genes and the TATA box (TATAAAAAA) is the most abundantly found example (Watson et al., 2014). The strong conservation of the core promoter across all protein-coding genes can be observed from its structure and binding factor whereas other upstream promoters varies in terms of binding factors and structures (Wray et al., 2003). Enhancers, on the other hand, are generally located in non-coding genomic regions where they are either sequence- or functionally conserved (or both) across different species (Levine & Tjian, 2003). The RNA polymerase II requires the interactions between the enhancers and promoters along with the recruitment of general transcription factors (TFIIA, -B, -D, -E, -F and -H) and chromatin remodelling complexes (RSF, PBAF, SWI/SNF and ACF) in order to initiate gene transcription (Watson et al., 2014).

Cis-regulatory modules like the enhancers play major roles in shaping the phenotypes of species evolutionarily as well as enabling numerous important biological processes such as morphogenesis and anatomy development to occur in an ordered manner without causing chaos like the cancer progression (Watson et al., 2014). The association between protein-coding regions and the onset of genetic diseases has been well studied throughout the decades, however it was not until recently that researchers start placing focus on the non-coding genomic regions and discover the effects of their variants on disease-related phenotypic differences (Kim et al., 2011; Visel et al., 2009).

Enhancer

The term 'enhancer' was first introduced by De Villiers and Schaffner (1981) to define a 72 bp DNA sequence repeat that can significantly activate the β-globin gene from rabbit. The proposed action of enhancer was described as element that could alter the superhelical density of DNA, facilitated the accessibility of RNA polymerase II and allowed for nuclear matrix binding (De Villiers & Schaffner, 1981).

Enhancers are short DNA elements with lengths ranging from 50 to 1500 base pairs, capable to serve as binding platforms for transcriptional activators such as transcription factors (Blackwood & Kadonaga, 1998). Upon binding of these activators, enhancer becomes functional and can elevate the transcription of gene it regulates to a much higher level. Enhancers are mostly cis-acting, they can be found up to 1 Mbp away either upstream or downstream from the gene (Blackwood & Kadonaga, 1998). The enhancer can function in both forward and backward directions of the DNA reads, making them more versatile than promoters in terms of their mode of activations. Some enhancers can even function in the form of enhancer-originating RNAs (eRNAs) where RNA polymerase II is recruited by enhancer itself and together with general transcription factors, eRNAs are transcribed (Melo et al., 2013; Natoli & Andrau, 2012). The eRNAs can significantly improve the efficiency of enhancers (Melo et al., 2013; Natoli & Andrau, 2012).

The first enhancer identified was the SV40 enhancer (Banerji et al., 1981) and this enhancer was found to be highly efficient in enhancing the expression of beta-globin gene in HeLa cell line. In their study, they first cloned the rabbit hemoglobin beta 1 gene isolated from rabbit and insert the gene into a recombinant expression plasmid containing SV40 enhancer. The recombinant plasmid containing SV40 enhancer had successfully produced 200-fold more gene transcripts as compared to plasmid without SV40 enhancer (Banerji et al., 1981). Banerji et al. (1981) concluded that this enhancer can improve gene transcription in both orientations and at any positions (1400 bp upstream or 3300 bp downstream from transcription start site) from the rabbit beta-globin gene. Since then, many enhancers in the human genome such as HACNS1, sensory vibrissae enhancer, forebrain subventricular zone enhancer and

penile spine enhancer have been discovered, characterized and deposited in various databases like the VISTA and FANTOM5 (Andersson et al., 2014; McLean et al., 2011; Visel et al., 2007).

The identification of enhancers can be conducted via two main approaches, namely the experimental and the computational approach. The experimental wet lab approach involves reporter assays (e.g. undirected integration of enhancer-reporter vector, enhancer trap and transient transgenesis assay) and high-throughput assays (e.g. STARR-seq and RNA-seq) in search for candidate enhancers that can activate the reporter gene (Arnold et al., 2013; Cao & Yip, 2016; Kvon, 2015; Mello et al., 1991; Patwardhan et al., 2009; Pennacchio et al., 2006; Schwarzer & Spitz, 2014; Visel et al., 2009). The disadvantage of the experimental wet lab approach is that the enhancer is usually tested on a context (in terms of disease states, developmental stages and chromatin states) different from the original context, making it hardly reflected on the 'actual' context in the living system (Cao & Yip, 2016). The computational approach on the other hand enables for a wider scope of enhancer identification on a genome-wide scale in a much shorter period and lower cost. There are various enhancer predictor tools such as GMFR-CNN, CSI-ANN, LS-GKM, DeepBind and iEnhancer-2L (Alipanahi et al., 2015; Firpi et al., 2010; Ghandi et al., 2014; Liu et al., 2015; Wong et al., 2016) that utilizes various gold standards of enhancer (namely histone modifications, transcription binding motifs, evolutionary conservation, DNA methylation and chromatin accessibility) to predict them from the genome (intergenic regions and non-coding regions) with various accuracies achieved (Cao & Yip, 2016; Lim et al., 2018a).

The link between coding regions and onset of numerous genetic diseases had been long established and that of the non-coding regions are picking up their paces. Throughout centuries, the search for the ultimate cure of genetic diseases in human via the genetic approaches such as gene therapy and gene editing faced various challenges and obstacles (Mubiru et al., 2008, 2011; Phillips et al., 2014). One of the major concerns is that human testing is restricted by ethical issues and human rights, therefore model organisms are normally used as test subjects beforehand before administration is to be done onto humans. There are many vertebrate model organisms such as the *Danio rerio* (zebrafish), *Mus musculus* (mouse), *Mesocricetus auratus* (golden hamster) and *Oryzias latipes* (medaka) being well studied to aid in the understanding of vital biological pathways and mechanisms leading to genetic diseases (Dooley & Zon, 2000; Fan et al., 2014; Lin et al., 2016; Perlman, 2016; Wittbrodt et al., 2002).

Recently, biomedical researches have been focusing on the potential of non-human primates as model organisms for disease studies and gene therapies. The advantage of using non-human primates as model organisms is that they share higher similarities with human in terms of genetic

contents, dietary factors, responses to environmental stimuli and even epigenomics (Huang et al., 2015). Besides, they also share physiological resemblances such as cognitive aging, reproduction, cognition, development and neuroanatomy with human (Phillips et al., 2014). Moreover, the primates are important disease models for primate-specific diseases such as AIDS as well as prostate diseases, lung malfunction syndrome and androgen receptor related diseases (Mubiru et al., 2008, 2011; Phillips et al., 2014). On the side note, non-human primates can be divided into a few categories, namely New World, Old World, prosimians, hominoids and Great Apes. Among them, the New World and Old World monkeys are more widely used as primate models (Phillips et al., 2014).

At earlier stages in primate epigenomic field, the functional epigenomic comparison studies among primates are restricted to lymphoblastoid cell lines only (Cain et al., 2011; Shibata et al., 2012; Zhou et al., 2014) and across 20 selected mammals this was conducted on the whole organ (liver) context (Villar et al., 2015). The histone modification H3K27ac (one of the enhancer mark) from different developmental stages of limb was compared across human, mouse and rhesus macaque (Cotney et al., 2013). Then, the FZD8 enhancer in the developing neocortex was examined for human and chimpanzee (Boyd et al., 2015). The iPSC of chimpanzee was also used as a model for the comparisons of neural crest cell enhancers in chimpanzee and human (Prescott et al., 2015). The abovementioned studies had proven that how enhancer variants across primates as well as absence or inactivity of enhancer strongly affected the speciation and divergence process during evolution. The milestones established by these previous studies are the stepping stones for the discovery of more functionally significant enhancers that are primate-specific and evolutionary significant.

A group of researchers had started to work on annotating the proboscis monkey genome and further predicted enhancers from the chromosome 18 of the genome using five different enhancer predictor tools (Omar et al., 2017). In their study, they utilized five different enhancer predictor tools (namely GMFR-CNN, CSI-ANN, LS-GKM, DeepBind and iEnhancer-2L) that use different epigenetic features (such as CTCF, EP300, HSK4me1, H3K4me3 and H3K27ac marks) as benchmark in identifying enhancers from the proboscis monkey genome (Alipanahi et al., 2015; Firpi et al., 2010; Ghandi et al., 2014; Liu et al., 2015; Omar et al., 2017; Wong et al., 2016). Omar et al. (2017) had consolidated the outcomes from the five enhancer predictor tools and found that the utilization of various epigenetic features in enhancer prediction had indeed improved the prediction power in general. Nevertheless, they leave the window open with the statement saying that some other epigenetic marks such as DNase I hypersensitivity, GATA1 and TAL1 are not included in their study, and enhancers in other chromosomes of proboscis monkey are yet to be explored in the future for evolutionary and medical studies contributing to larger scientific discoveries in future.

An interesting study on liver specific enhancers in human across various ethnics by Kim et al. (2011) had shown that the enhancer variants that were found across different ethnics might contribute to differing drug responses and thus this might provide the ultimate solution to the adverse drug events that had caused high mortality in this modern era. It has been widely known that enhancer variants can lead to several genetic diseases (Kleftogiannis et al., 2015), discovering these enhancer variants in endangered ancient primates like the proboscis monkey would greatly drive future conservation research.

In this review, the proboscis monkey was chosen as one of the potential primate candidates because of several unique aspects it possesses that are clearly distinctive from the other non-human primates known to date (which will be discussed in detail in the following sections) and the strong needs to protect it from the brink of extinction.

Proboscis Monkey

The proboscis monkey (*Nasalis larvatus*), also known as the long-nosed monkey, is one of the Asia's largest native monkey species. It is an Old World Monkey belonging to the Cercopithecidae family and it is exclusively endemic to Southeast Asian Island of Borneo. This monkey species is currently listed as endangered by IUCN (Meijaard et al., 2008). This reddish-brown skin-coated monkey can be easily distinguished from other monkeys via their unique morphology and appearance: their large and fleshy nose with growth capacity up to 7 inches in length (Harding, 2013). The proboscis monkey has grey limbs and large pot-shaped bellies. The sexual dimorphism is very distinctive where the size of the male is twice as big as the female in terms of head-body length and weight (Harding, 2013). The proboscis monkey possesses unique external nasal cartilages to support its huge nose (Maier, 2000) and it is the only member in the colobine genus that owns a narrow, cercopithecine-like interorbital pillar (Delson, 1994).

The proboscis monkey commonly survives in groups of females and one dominant male together with their young (Bennett & Gombek, 1993; Boonratna, 1993, 2002). Groups of some males and the all-males group had also been reported (Boonratna, 1999; Murai, 2004). The social interactions in the group of around 9-60 individuals are mostly peaceful with minor aggressions (Bennett & Gombek, 1993; Boonratna, 1999; Yeager, 1992). The natural habitats of the proboscis monkeys are mainly riverine, dipterocarp and swamp forests (Bennett & Gombek, 1993). This monkey is one of the best swimmers among non-human primates despite the fact that they live most of their lives on trees foraging for flowers, insects, leaves and fruits (Boonratna, 1993; Sebastian, 2000). On average, they could live up to fifteen to twenty years (Harding, 2013).

Significance of Proboscis Monkey in Enhancer Studies

The booming effects coming from the rise of the next generation sequencing had pathed the way for the completion of genome

sequencing in various primates (e.g. rhesus monkey, marmoset, bonobo, gorilla and orangutan) (Gordon et al., 2016; Prüfer et al., 2012; The Marmoset Genome Sequencing and Analysis Consortium, 2014). Likewise, there are also some on-going primate genome sequencing projects on drill, mouse lemur, sooty mangabey, gibbon, baboon, white and black colobus, sifaka lemur and owl monkey (Baylor College of Medicine-Human Genome Sequence Center [BCM – HGSC], 2016). Above all, the recently sequenced genome of the proboscis monkey is the one primate genome that is stepping new into the limelight (Abdullah et al., 2014). This primate genome is essential for the opening of a new window for the genome-wide discovery of enhancers for several reasons.

The unique morphological traits of the proboscis monkey are one of the reasons it is viewed as the most primitive primate. From the Pleistocene to the Holocene, the Asian colobines are exposed to extensive adaptive radiation in which they are subjected to a vast range of adaptations and selection pressures in numerous environments with differing altitudes, climates and vegetations (Davies, 1994). These adaptations have interestingly introduced a myriad of diversity in terms of differentiation in the structures of the body and social behaviours, the speciation process that is still progressing till today (Davies, 1994). Peng et al. (1993) had conducted classification of Asian colobines (on 123 skulls) based on 14 characteristics and further deduced the morphology-based evolutionary phylogeny. The traits of colobine monkeys (namely *Nasalis*, *Pygathrix*, *Prebytis*, *Rhinopithecus* and *Presbytiscus* (*Rhinopithecus avunculus*)) are subjected to discriminant, one-way and cluster analyses (Peng et al., 1993). In most of the cases, the dentition, cranial skeleton, cranial morphology as well as the general anatomy are the major discriminant factors among the colobine genera. Moreover, Peng et al. (1993) proposed the possibility of the proboscis monkey of being a primitive based on features it shared with the *Rhinopithecus*: skull structure, highly-distinctive sexual dimorphism, terrestrial movement and proportions of the extremities. Thus, these morphological characteristics of the proboscis monkey may suggest that it may belong to one of the long-isolated genera within the colobines (Giusto & Margulis, 1981; Groves, 1989; Peng et al., 1993). Morphological characteristics such as brain, skull and facial structure are governed greatly by enhancers, and essentially it is believed that these enhancers are vital contributors towards primate evolution. For instance, the human HARE5 (human-accelerated regulatory enhancer) displayed dramatic performance as compared to that of chimpanzee in corticogenesis and neural progenitor cell cycle in developing neocortex; the brain size of transgenic mice is also much bigger with the presence of this enhancer from human in contrast to that of chimpanzee (Boyd et al., 2015). Besides, genes associated with enhancer divergence in both neural crest of human and chimpanzee are enriched with species-biased enhancers, indicating the potential

of enhancers in orchestrating the facial morphological variations between both primates (Prescott et al., 2015). Therefore, the ancient morphological characteristics such as the nose and skull structure of the proboscis monkey is an indication of a unique enhancer landscape that is yet to be explored.

The proboscis monkey is unique among other primates in terms of their digestive physiology. Like other colobine monkeys, the proboscis monkey is a foregut fermenter and it possesses overdeveloped salivary glands that are capable of high saliva production (Bigoni et al., 2003). Their large stomachs are four-chambered and are responsible for cellulose digestion especially in the forestomach where symbiotic microorganisms are abundant in amount. The first two stomach chambers (*presaccus* and *saccus gastricus*) allow for actions of symbiotic microbiota to disintegrate cellulose with saliva as pH buffer whereas the other two stomach chambers (*tubus gastricus* and *pars pilorica*) digest the bacteria using numerous digestive enzymes (Oates et al., 1994). Interestingly, adaptive convergence was found to occur between the lysozyme of colobine monkey and that of the ruminant, thus marking the striking differences between the lysozyme of colobine monkeys and that of the mammalian (including that of human) lysozyme (Stewart et al., 1987). The dietary habits of a host organism have substantial effects on its epigenetic regulations as well as developmental origins of health and diseases (Mochizuki et al., 2017). Generally, the methylation of histones and DNA (the key player in epigenetics) are affected by any bioactive elements or conditions that can influence the AdoHcy (S-adenosylhomocysteine) and AdoMet (methyl donor of methylations) levels in the host (Choi & Friso, 2010). A total of 738 species-specific genes were discovered from the whole genome of the proboscis monkey where genes such as the expanded SusE outer membrane protein (PF14292) and glycogen synthase I (*GYSI*) gene are associated with starch utilization (Tamrin, 2016). In addition, the sweet taste receptor *Tas1r2* gene of the proboscis monkey was found to be greatly diverged from all its other anthropoid primate counterparts, which explains for the dietary shift in this species (Tamrin, 2016). Besides, the gut microbiome also plays part in orchestrating the epigenome (in terms of chromatin modelling and DNA alterations) via the synthesis of low molecular weight byproducts that eventually contribute to the DNA methylation process (Lewis & Tollefsbol, 2017). The unique gut microbiome and dietary of proboscis monkey are another two key reasons for the need for enhancer studies in this species because it is interesting to discover how the dietary habits of ruminant in primates affect the enhancer landscape as whole. This is one of the adaptive traits we wish to explore in endangered wildlife like the proboscis monkey and further improve our comprehension on how this unique epigenome of proboscis monkey is associated with the enhancer landscape they possess (Luo & Lin, 2016; Vogt, 2017).

The karyotypes of mammals and primates were extensively investigated by Müller (2006) in search for the ancestral primate karyotype. In his study, he discovered that the differences between the inferred ancestral mammalian karyotype (2n=2x=46) and ancestral primate karyotype (2n=2x=50) were small with fusions and fissions involving chromosome 4, 8, 10, 12 and 22 (Müller, 2006). Of all prosimians studied, the primitive karyotype is not present, and their karyotypes are highly derived. The karyotype diversity of the New World monkeys is greatly varied with a wide range of chromosome numbers from 2n=2x=16 to 2n=2x=62, the inferred ancestral karyotype for this group is 2n=2x=54. Among the New World monkeys included in the study, only the common marmoset (*Callithrix jacchus*) and Pygmy marmoset (*Callithrix pygmaea*) have chromosome number strongly conserved to that of human, which is 2n=2x=46 and 2n=2x=44 respectively. The hominoids studied depicted diverse karyotype ranging from 2n=2x=38 to 2n=2x=52 with *Hylobates* (2n=2x=44) having the closest karyotype to that of human. All the Old World monkeys investigated generally have strong conserved karyotypes with the exception of African green monkey (*Chlorocebus aethiops*) (2n=2x=60) and *Cercopithecus wolfi* (2n=2x=72). The baboons and macaques share the same chromosome number (2n=2x=42) whereas leaf-eating monkeys like the white and black colobus (*Colobus guereza*) possess chromosomal number of 2n=2x=48. The proboscis monkey was reported to possess chromosome number of 2n=2x=48 which was considered fairly conserved compared to human (Chiarelli, 1966; Soma et al., 1974; Stanyon et al., 1992). In short, in the selection of suitable primate model organism that have conserved karyotype to that of human, the common marmoset and Pygmy marmoset has the highest potential among the New World monkeys whereas the proboscis monkey and the white and black colobus are among the most feasible Old World monkey candidates. Comparing the chromosome of the proboscis monkey with that of human, a reciprocal translocation followed by pericentric inversion had led to the events of fragmentation and association of human chromosome 1 and 19 onto chromosome 5 and 6 of proboscis monkey (Bigoni et al., 2003). Chromosomal translocation can sometimes change gene expression and enhancer functioning that favours the overexpression of oncogene when oncogene is proximity to strong enhancer of other gene, thus causing cancer (McNeil et al., 2003). The chromosomal dissimilarities between human and proboscis monkey, when added with the knowledge on the enhancer landscape of this non-human primate, would be a big advantage for us to discover the adaptive traits of this wildlife. Moreover, this is also essential for future decisions on the best effective medical treatments and optimal drug dosage for species conservation.

FUTURE PERSPECTIVES

The genome-wide enhancer identification in proboscis monkey is a trend we foresee happening in the near future as we discussed in the previous sections. From its various morphological characteristics that suggest that it is one of the most primitive primates belonging to a long isolated genera, to its one-of-its-kind cytogenetics as well as its dietary habits, these had spiked our interest in understanding the epigenetic and enhancer landscape of this species. -

The importance of enhancer landscape discovery in the proboscis monkey can be seen in terms of how they can be used to aid in conservation measures and disease treatment in future. Now that the genome of the proboscis monkey had been sequenced, we can finally conduct an epigenome comparison between this species and that of human to discover the uniquely ancient and unevolved enhancer landscape. This serves as an important milestone to identify the enhancer variants in proboscis monkey that are disease-causing and further plan on the strategies to conserve this species via conservation research like antibody synthesis against elephant endotheliotropic herpesvirus (EEHV) (Kochagul et al., 2018) and pathogen combatting in white-nose syndrome in bats (Palmer et al., 2018). Besides, the proboscis monkey enhancer landscape is useful in the understanding of the adaptive phenotypic traits that occur in the environment for displaced wildlife (Luo & Lin, 2016; Vogt, 2017) for more effective conservation measures and strategies in future.

In a nutshell, the potential of the proboscis monkey in the role of providing us with the most primitive enhancer landscape is undeniably huge in the near future. It is now essential for consolidation of efforts in the discovery of enhancers from the genome of the proboscis monkey and further characterize them functionally to enhance our understanding on the onset and treatment of some genetic diseases accounted by enhancer variations across the proboscis monkey and that of human.

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PCR-RFLP Characterization of Major Histocompatibility Complex (MHC) *B-LβII* Gene in Nigerian Locally Adapted Chickens

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ABSTRACT

DNA polymorphism at *B-LβII* region of chicken Major Histocompatibility Complex (MHC) was studied in Nigerian locally adapted chicken genotypes, namely: Normal-feathered, Frizzled-feathered and Naked neck with much less history of selection for economic traits, though, previously selected for response to sheep red blood cell (SRBC). Response to SRBC (1% suspension, i/v) was estimated by haemagglutination (HA) test 5 days post inoculation (dpi). Thereafter, high and low responding groups were generated. DNA was isolated from high and low responders at second generation of selection as well as randomly selected individuals from the locally adapted chicken population. The *B-LβII* was amplified with specific primers and an amplicon of 277 bp obtained in each sample was digested with three restriction enzymes (RE) viz., *Msp*I, *BseR*I and *Taq*I individually. While the PCR-RFLP of B-LβII with *Msp*I and *BseR*I had no cutting sites, *Taq*I RE exhibited monomorphic pattern with genotypes AA and at frequency 1.0 in the divergently selected groups. The Nigerian locally adapted chicken is known for high disease resistance. The monomorphic could be as a result of fixation of naturally selected genotype AA.

Keywords: *B-LβII*, Nigerian locally adapted chicken, PCR-RFLP, SRBC

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INTRODUCTION

The gene region of the genome of the animal responsible for the resistance/ susceptibility to genetic disease was first identified as the target for graft rejection between individuals (Actor, 2007). The first completed Major Histocompatibility

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Complex (MHC) genomic sequence of a non-mammalian vertebrate was the chicken MHC (historically termed *B* locus and now MHC-*B*) (Kaufman et al., 1999). MHC is a gene region consisting of a sequence of genes encoding the MHC molecules and glycoproteins. They are arranged on the surface of cells and play a basic role of interaction among cells in the immune system (Abbas et al., 2000; Actor, 2007). The primary function of most of these molecules is the presentation of fragments protein antigens (epitopes) on effector cells of the immune system from which all chain immune responses develop (Abbas et al., 2000). The MHC genes are highly polymorphic varying widely between individuals, making each individual to have a particular variable efficiency in the presentation of different peptides. Thus, the host immune response particularly depends on their MHC (Frank, 2002).

The MHC plays a crucial role in the immune system. MHC region in chicken is often called B complex and extends on part of microchromosome 16. The chicken class II-β genes, located on locus B are referred to as *B-LβI* and *B-LβII*. Chicken MHCBL genes encode molecules that are similar to the classical MHC class II of its mammalian counterparts located on the surface of antigen-presenting cells including macrophages, and dendritic and B cells (Davison, 2008). They are involved in the antigen-presenting identification process initiated by T cells (Erf, 2004; Steinman, 2007) and also the interaction between T and B cells (Lamont, 1989) for the development of adaptive immunity. A high level of polymorphisms in *B-LβI* and *B-LβII* exon 2 that codes for the β1 domain in the antigen-binding region has been reported (Goto et al., 2002; Hosomichi et al., 2008; Jacob et al., 2000; Worley et al., 2008). This, in turn, greatly enriched the antigen types being recognized by the MHC class II molecules. Therefore, *B-LβI* and *B-LβII* are believed to be associated with resistance or susceptibility to many diseases, such as Marek's Disease (MD) (Niikura et al., 2004) and salmonellosis (Liu et al., 2002; Zhou & Lamont, 2003).

The *B-LβI* and *B-LβII* genes of the five genes present in this class B complex, are the main expression. The other three, the *B-Lβ* have low or no expression (Kaufman & Salomonsen, 1997). Therefore, the *B-Lβ* genes, along with *B-Lα* are responsible for, or at least primarily responsible for the presentation of different exogenous pathogens (extracellular parasites and bacteria) to the effector cells of the immune system of chickens, hence, for triggering the immune response to these agents in these organisms (Kaufman & Wallny, 1996; Trowsdale, 1995). Since the chicken *B-Lα* gene is monomorphic, but the β chain genes, especially the nucleotides that encode the peptide-binding region are polymorphic (Jacob et al., 2000), the differences presented by class II molecules, which are responsible for the variation in the immune response, are due to different alleles of *B-Lβ* genes present in their genome.

Considering the genetic potential of the Nigerian locally adapted chicken in terms of disease resistance, it is therefore necessary

that this research be conducted to investigate the polymorphism in the MHC class *B-LβII* gene in Nigerian locally adapted chickens.

MATERIALS AND METHODS

Experimental Animals and Procedure

Two hundred and seventy-nine local chickens comprising naked neck, frizzled-feathered and normal feathered generated from parents previously selected for high and low response to sheep red blood cells were used for the study. The chicken populations were representative from the six south-western states in Nigeria. Namely; Ogun, Osun, Ondo, Oyo, Ekiti and Lagos. All the experimental chickens were wing tagged and maintained under identical management conditions. Chickens were fed *ad libitum* with standard feed. Clean water was provided *ad libitum*.

Response to SRBC was assayed in the individual chickens at 8 weeks of age. Blood was withdrawn under aseptic conditions from healthy sheep and was used to make 1% suspension of SRBC. Each chicken was injected with 1ml of 1% SRBC via the jugular vein. Hyper immune sera was collected from individual birds 5 days post inoculation (dpi). The response to SRBC was assayed by HA test (Siegel & Gross, 1980). Reciprocal of highest dilution of antigen which showed complete agglutination was considered as HA titre.

Genomic DNA was extracted from the chicken erythrocytes using Qiagen tissue and blood DNA kit in accordance with the manufacturer's instruction. The quantity and purity of the DNA was checked on NanoDrop Lite Spectrophotometer and only good quality DNA samples were used for further analysis.

The following specific primers were used for the amplification (forward CTGCCCGCAGCGTTCTTC; reverse TCCTCTGCACCGTGAAGG) (Goto et al., 2002). PCR was carried out in final reaction volume of 25 µl. Each reaction volume contained of 1XPCR buffer, 1.5 mM MgCl2, 200 µM of dNTPs 10 pmole of each primer, 1U of Taq DNA polymerase, 50 ng of template DNA. Cycling conditions included initial denaturation at 94^{o}C for 5 minute followed by 35 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 58.3^{o}C for 1 minute and extension at72^{o}C for 1 minute and then the final extension at 72^{o}C for 10 minute. The presence of desired amplicon was confirmed by running the amplified PCR products (approximately 10 µl) on 1% agarose gel.

The PCR products were subjected to restriction analysis using the 3 restriction enzymes viz., *Msp*I, *BseR*I and *Taq*I separately under manufacturers' recommended assay conditions for 15 minutes. The digestion was performed in 20 µl using 10 µl PCR products, 1 U *Taq*I, 1X RE buffer and nuclease free water up to 20 µl. The digested products were resolved on 3% agarose gel at a constant voltage of 8 V/cm. The molecular sizes of the amplicon and digests were estimated with the help of molecular size ladder.

RESULTS AND DISCUSSION

Specific amplification of MHC *B-LβII* region resolved an amplicon of 277 bp (Figure 1) in divergent groups. The same region had been amplified as 235 bp and 267 bp by Livant et al. (2001) and Zheng et al. (1999). Though the primers used by them were different and were degenerate primers. PCR-RFLP analysis of *B-Lβ II* region in Nigerian locally adapted chickens was digested with three restriction enzymes, *Msp*I, *Taq*I and *BseR*I in order to detect polymorphism in the region among the Nigerian locally adapted chicken genotypes. Whereas *BseR*I (Figure 2) and *Msp*I (Figure 3) had no restriction sites, *Taq*I (Figure 4) cut at 116 bp and exhibited two bands of 116 bp and 161 bp. However, *Taq*I restriction enzyme (RE) used for PCR-RFLP analysis of *B-LβII* region in Nigerian locally adapted chickens showed monomorphic banding pattern with genotype AA at frequency 1.0. The monomorphic pattern indicated a conserved *Taq*I site in Nigerian locally adapted chicken population. However, Laxmanan and Lamont (1998) and Weigend and Lamont (1999) had reported differences in high and low lines selected on the basis of multi-trait index by RFLP analysis using *Pvu*II and *Sac*I digest. The present result is in agreement with the report of Sivaraman et al. (2005) who observed monomorphic banding pattern in the PCR-RFLP of 267 bp *B-Lβ II* family of MHC region with all three restriction enzymes employed which included *Taq*I and *Msp*I. Ahmed et al. (2008) also reported monomorphic pattern in *Taq*I PCR-RFLP in Synthetic Dam Line broiler chicken lines divergently selected for SRBC and cell-mediated immunity responses for one generation. Sivaraman and Kumar (2005) obtained a monomorphic PCR-RFLP pattern of the same region in White Leghorn chicken with *Taq*I RE. Weigened and Lamont (1999) denoted that most of the polymorphism found in the MHC region were in tenth generation of multi-trait immunocompetence divergent selection and suggested that their genetic background rather than divergent selection were responsible. Ahmed et al. (2007) also reported monomorphic PCR-RFLP profile of similar region that was, exon 2 of *B-LβII* gene in turkey.

The Nigerian locally adapted chicken is known to be well adapted to the tropics and resistant to most tropical diseases. Therefore a monomorphic banding pattern with genotype AA and a frequency of 1.0 indicated that the naturally selected genotype AA might have been fixed in chicken. Furthermore, the absence of polymorphism according to Sivaraman et al. (2005) probably might be due to the fact that two generations of divergent selection for Immunocompetence index were not sufficient to generate or accumulate DNA polymorphism at *B-LβII* family of MHC region, that could be detected by PCR-RFLP with the enzyme employed or there was no variation in the RE sites. To further buttress the result of this study, Singh (2008) reported that *Taq*I AA genotype showed high frequency in guinea fowl while in chicken both AA and AB genotype showed almost equal frequencies. This suggested

that guinea fowl used in their experiment showed less heterozygosity as compared to the chicken. This was so since the guinea fowl used in their experiment represented an almost unselected flock while the chicken population that is, Synthetic Male Line was a synthetic population, and hence homozygosity was expected in guinea fowl in comparison with Synthetic Male Line chicken population.

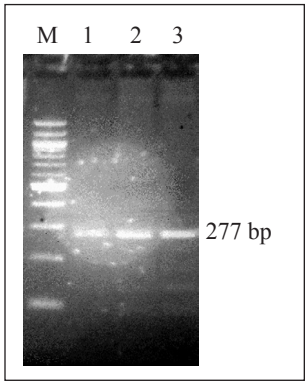

Figure 1. A PCR-amplified MHC *B-LβII* gene from Nigerian locally adapted chicken genotypes. Lane M= DNA marker (100 base pairs) lanes 1-3 show amplicon of 277 bp

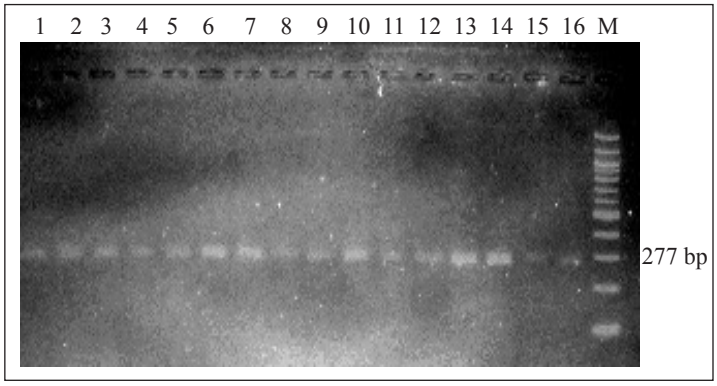

Figure 2. *Bse*RI PCR-RFLP in *B-LβII* gene of Nigerian locally adapted chicken genotypes divergently selected for antibody response to SRBC. Lane M= DNA marker, *Bse*RI digest, lanes 1-16

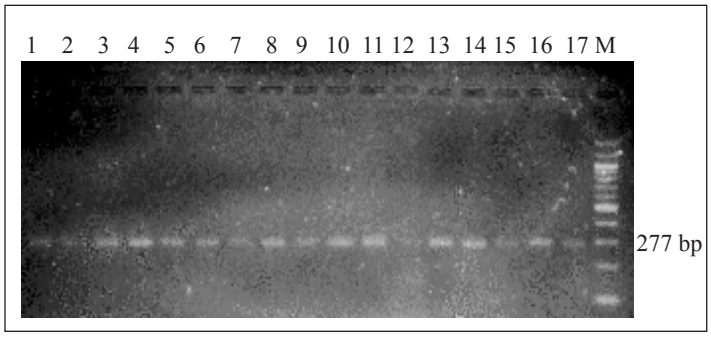

Figure 3. *Msp*I PCR-RFLP in *B-LβII* gene of Nigerian locally adapted chicken genotypes divergently selected for antibody response to SRBC. Lane M= DNA marker, *Msp*I digest, lanes 1-17

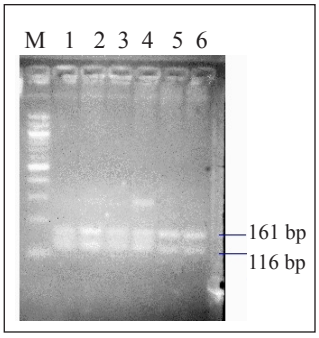

Figure 4. *Taq*I PCR-RFLP in *B-LβII* gene of Nigerian locally adapted chicken genotypes divergently selected for antibody response to SRBC. Lane M= DNA marker, *Taq*I digest, lanes 1-6

CONCLUSION

The Nigerian locally adapted chicken genotype is well adapted to the tropical conditions and as such is believed to be resistant to most of the tropical diseases. The monomorphic banding pattern with genotype AA and frequency 1.0 indicated that the naturally selected genotype AA might have been fixed in this group of chicken and could be associated with their ability to survive in the Tropics. The result obtained for *Taq*I restriction enzyme which had two restriction sites for the *B-LβII* region showed that this restriction enzyme could be used as a tool for the evaluation of polymorphism in the MHC of Nigerian locally adapted chicken.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Biorisk Assessment of Antibiotic-Resistant Pathogenic Bacteria Isolated from Swiftlet Houses in Sarawak

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ABSTRACT

The occurrence of antibiotic resistance in pathogens is a growing concern globally. Development of multiple antibiotic-resistant bacteria has overwhelmed new medical advancement and threatens patients with untreatable infections. The qualitative risk assessment study was carried out to investigate the relative effects of the main determinants of antibiotic-resistant pathogenic bacteria and to estimate the risk of the emergence and spread of antibiotic resistance among humans in the swiftlet's faeces and its indoor air to human health. The methodology applied focused mainly on three main sections namely the hazard identification, exposure assessment, and hazard assessment. Sources of data for bio risk assessment include published literature, data from on-going research projects and data collected from the industry. The results showed that the prevalence of isolating Gram-positive bacteria were higher in swiftlet houses. Over half of the pathogenic bacterial isolates were multidrug-resistant to a wide range of commonly used antibiotics such as *Bacillus, Enterococcus*, *Escherichia coli*, *Staphylococcus*, *Lysinibacillus*, *Paenibacillus and Sporosarcina*. 80% of the bacteria isolates showed high MAR index of over 0.2. These emerging pathogenic

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antibiotic resistant bacteria are capable of causing life-threatening infections which pose a health hazard to our biodiversity.

Keywords: Airborne, antibiotic resistant, bacteria, faeces, pathogenic, risk assessment

INTRODUCTION

The increase of human population and uncontrolled development of industrial and agricultural expansion in developing country has led to the occurrence of public health problems such as multi-antibiotic resistant pathogenic bacteria which pose serious health hazards to the human society (Bartoloni et al., 2004). Risk assessment is commonly used to assess the risk in the environment, engineering industry, military industry, food safety and public health services (Lacey, 2011). Risk assessment is defined as the identification of the risks or hazards in certain environment qualitatively and quantitatively. Risk assessment focuses mainly on the quantification on the probability of harmful effect towards individuals or populations from certain human activities (Lerche & Walter, 2006).

Swiftlet industries have sprouted spontaneously across the whole Peninsular Malaysia within a few years due to multi-million dollar potential profit. The waste from the birds especially the faeces is usually used as organic fertilizer in the agricultural industry because of its rich nutrient, water, nitrogen, phosphorus, potassium and minerals content. Most bacteria are able to grow in the bird faeces and the air inside the bird-house. The emergence of antibiotic-resistant bacteria may contaminate vegetables through this bird waste fertilizer. Improper washing of these contaminated vegetables may also be cross-contaminating into our foods in the kitchen, causing harmful diseases to human. Intensive and extensive abuse of antibiotics has caused the formation of an antibiotic-resistant genes pool in the environment. There is a growing concern regarding public health hazards and implications due to the presence of antibiotic residues in the environment and their effects on the development of pathogenic bacterial resistance from these bacteria in the swiftlet faeces. Serious action needs to be taken because there are cases of antibiotic-resistant pathogenic bacteria detected in drinking water (Xi et al., 2009), vegetables, fish (Kabir et al., 2014; Schmidt et al., 2001), healthy adults, food animals, foods and the food industry (Boonyasiri et al., 2014).

Risk assessment is normally focused on all microorganisms isolated from an environment and the results can provide a framework for risk management to minimise the health hazards to human (Michael, 2008). Several pieces of research have demonstrated that cases of increased bacteria resistance to biocide are rising sharply (Langsrud et al., 2003; Walsh et al., 2003). According to the literature, so far no study has been carried out to assess the risk of potential health hazards to human and environment caused by the pathogenic bacteria from the swiftlet houses. The

emergence of the pathogenic antibiotic-resistant bacteria in both humans and food animals is a growing concern to the public health. Therefore, the present study was carried out to investigate the emergence of resistance in any antibiotic-resistant pathogenic bacteria and the subsequent risk to human health relating to specific bacterial isolates from the swiftlets' faeces and indoor air of the swiftlet houses.

MATERIALS AND METHODS

Sampling Sites

Swiftlet houses selected for the present study were located in Kuching (01°32'56.6''N 110°22'27.5''E), Kota Samarahan (01°27'34.2''N 110°27'25.9''E), Semarang (01°40'40.0''N 111°6'5.92''E), Maludam (01°39'14.17''N 111°1'53.9''E), Sepinang (01°40'11.8''N 111°7'5.9''E), Betong (01°24'0''N 111°31'0''E), Saratok (01°44'10.32''N 111°21'10.22''E), Sarikei (02°6'3.75''N 111°30'39''E), Sibu (02°19'11.3''N 111°49'50.5''E) and Miri (04°23'39.2''N 113°59'12.2''E).

Sources of Bacterial Isolates

Faecal and Airborne Bacteria Collection. A Total of 1200 faecal and airborne bacteria isolates was selected from a collection of strains obtained from previous studies (Leong et al., 2013a; Leong et al., 2013b). Five faeces samples were collected randomly from the floor of each swiftlet house of the sampling site. The faeces sample was then plated on nutrient agar (Merck, Germany) plates in duplicate and incubated at 37± 1° C for 24 hours. The collection of the indoor airborne samples procedures was carried out according to Department of Veterinary Services (DVS) (2017). The airborne bacteria were obtained using exposed plate count agar (Scharlau, Spain) in duplicate. The lid of the plates was lifted and exposed in the air for 15 seconds inside the swiftlet house. The plates were incubated at 37± 1°C for 24 hours in the laboratory. The bacteria isolates were further identified by using biochemical tests. Species identification was confirmed using 16S rRNA sequencing.

Biorisk Assessment

The biorisk assessment was performed according to the European Agency for Safety and Health at Work (EU-OSHA) (2000), European Parliament and Council directive (EP) (2000), Health Protection Agency (HPA) (2007), Jeenaa et al. (2006) and Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) (2009) with modification. The methodology of biorisk assessment applied in this study focused mainly on the three main sections namely the hazard identification, exposure assessment, and hazard assessment. Sources of data for biorisk assessment of pathogenic bacterial resistance problem include published literature, data from on-going research projects and data collected by the industry.

Hazard Identification and Exposure Assessment

In relation to the antibiotic-resistant pathogenic bacteria, the microbiological

hazard may be a pathogen with resistance to a particular antimicrobial. Based on the results reported by Leong (2015), the prevalence of each pathogenic antibiotic-resistant bacteria species was determined. Collecting the results from past researches provides an insight into the understanding of the bacterial diversity and how frequently these bacteria are detected in the faecal and airborne samples of the swiftlet houses.

Human Health Risk Assessments

Predisposition of Bacterial Species to Acquire Resistance and Pathogenicity. Bacterial species are categorised according to the assessment procedures described in HPA (2007) and SCENIHR (2009). All the available scientific information and literature evidence of the bacterial collection were assessed according to the following potential risk:

High: *Enterococcus* and *Enterobacteriaceae* bacteria poses high occurrence frequency and specialised mechanisms in horizontal gene transfer. Thus, the probability of pathogenic or resistance gene exchange between related or unrelated species is high among these bacteria genus.

Medium: *Lactococcus* bacteria are susceptible to the intra-generic gene transfer mechanisms. *Lactococcus* poses high-frequency in conjugal transfer of plasmid-encoded pathogenicity and resistance gene. Thus, *Lactococcus* carrying such inherited high-frequency conjugation systems may pose a medium risk to human health.

Low: *Bacillus* bacteria are endospore-forming bacteria which may not have any clear mechanism of conjugation. Horizontal spread of the virulence plasmids is not unusual, thus *Bacillus* still pose a low risk to human health.

Antibiotic Susceptibility Testing and Multiple Antibiotic Resistance (MAR) Indexing of The Isolates. The antibiotic susceptibility testing and MAR indexing were performed according to Leong et al. (2013b). The faecal and airborne bacterial isolates were tested using the disc diffusion method against 23 commonly used antibiotics. The antibiotics impregnated discs and the recommended concentrations for use in this testing were as follows: chloramphenicol (30µg), ampicillin (10µg), tetracycline (30µg), streptomycin (10µg), gentamycin (10µg), erythromycin (15µg), cephalothin (30µg), nitrofurantoin (300µg), tobramycin (10µg), rifampin (5µg), kanamycin (30µg), sulphamethoxazole/ trimethoprim (1.25/ 23.75µg), amikacin (30µg), imipenem (10µg), ceftriaxone (30µg), penicillin G (10U), doxycycline (30µg), ceftazidime (30µg), norflaxacin (10µg), vancomycin (30µg), pipercillin (100µg), ciprofloxacin (5µg) and nalidixic acid (30µg). The MAR index for each bacterium isolated was determined using the method described by Tambekar et al. (2008). A value greater than 0.2 indicated that the culture was MAR and the number of the MAR index for an antibiotic indicated its sensitivity and resistance of certain bacteria to antibiotics. The use of MAR index is to assess the potential health risk.

Pathogenic Gene Detection. Multiplex-PCR was applied in the molecular detection of various pathogenic genes in *E. coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Bacillus cereus* to assess their hazardous risk to human health. The *Escherichia coli* O157:H7 (EDL933 strain), *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 11778 and *Escherichia coli* ATCC 25922 were used as positive control respectively for the detection of virulence genes. Specific primer sequences were synthesized in order to identify common pathogenic bacteria. The PCR was performed in the detection of Shiga toxin (*stx* gene) in *E. coli*, *opr*L gene in *P. aeruginosa*, virulence genes (*ace*, *AS*, *efaA*, *gelE*) in *E. faecalis* and enterotoxigenic genes (*hbla*, *entFM*) in *B. cereus*.

Exposure Hazards. The processes that lead to the spread of these pathogenic antibiotics resistant bacteria are described. These bacteria may release, survive and even exposed to the environment, human, animal, plant or other microorganisms, leading to a more serious problem in the future. The exposure assessment identified the reservoirs from which this antibiotic resistance pathogen can emerge. Evaluation of how, and how much, a person, or a population is exposed to the hazard(s) was assessed.

RESULTS

Hazard Identification

The bacteria isolates were confirmed using 16S rRNA sequencing. The agarose gel electrophoresis banding pattern was shown in Figure 1.

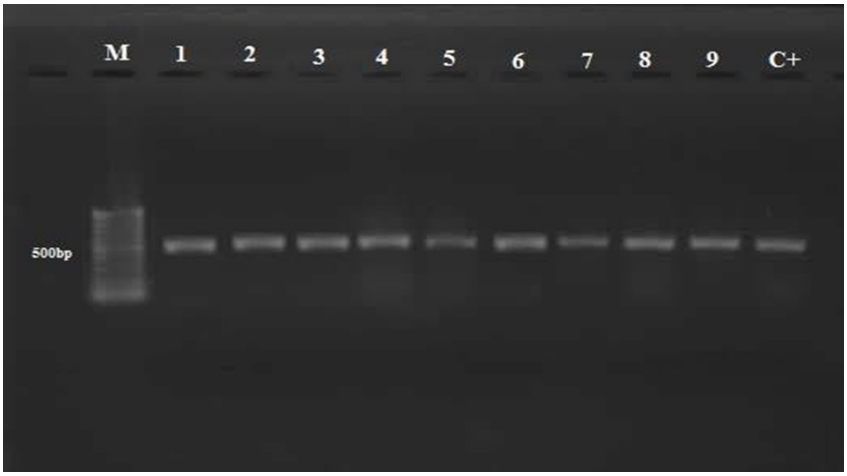

Figure 1. Agarose gel electrophoresis of 16S rRNA sequencing gene amplification products for bacteria isolates. M: 100bp ladder; 1-9: Bacteria isolates; C+: Control

Antibiotic Resistance and MAR Pattern

The results showed pathogenic bacteria isolates such as *Bacillus* spp., *Enterococcus* spp., *E. coli*, *Staphylococcus* spp., *Lysinibacillus* spp., *Paenibacillus* spp. and *Sporosarcina* spp. Had developed antibiotic resistance to a wide range of commonly used antibiotics (Tables 1 and 2). Gram-positive bacteria were the most highly prevalent bacteria found in all the swiftlet houses. 96% and 80% of the Gram-positive faecal and airborne bacteria respectively showed resistance to more than 2 antibiotics tested. *Escherichia coli* were resistant up to 10 types of antibiotics tested with MAR index over 0.47. *Bacillus pumilus* was resistance to all antibiotics tested. MAR indexing of the isolates and elucidation of the resistance patterns revealed that the faecal and indoor airborne bacteria isolates showed high-level MAR index ranged from 0.05 to 1.0.

Pathogenicity

The pathogenicity of the bacteria isolated was discussed in Table 1 and Table 2. Most of the bacteria showed different levels of pathogenicity to human, plants, insects or even animals.

Exposure Assessment

The processes that lead to the acquisition, selection, and spread of the resistant pathogenic bacteria and genes in humans are shown in Table 3. The exposure assessment was assessed for the four main reservoirs (human, plants together with insects, animal and environment) from which antibiotic resistance pathogen could emerge. If these antibiotic-resistant bacteria are exposed, they may cause medium to high risk to human and ecosystem.

DISCUSSION

Bacterial isolates resistant to more than two antibiotics were considered as having multiple antibiotic resistance, accounted for approximately over 80% of the total isolates encountered in the present investigation. Most of these isolates had resistance indices ranging from 0.05 to 1.0. The occurrence of MAR bacteria in the environment is certainly a well-known phenomenon. Many investigators believe that these drug-resistant organisms have become more common recently due to the extensive use of antibiotics in medicine and agriculture throughout the world (Barbosa & Levy, 2000). Apart from variations among the different genera, wide variations in the MAR index and resistance patterns were also noticed within the different strains of the same genera, indicating the diversity of the strains. While maximum variation in the MAR index and resistance patterns were observed among the different strains of *Bacillus* and followed by *Staphylococcus*. This is indicative of a common source of *Staphylococcus* contamination. Studies by Becker et al. (2014) revealed that *Staphylococcus* represented contaminating bacteria.

The results obtained in this study revealed bacteria with multiple antibiotic resistance patterns suggesting the possible horizontal gene transfer among non-related

Table 1

Health risk assessment of the potential antibiotic resistant pathogenic faecal bacteria isolated from the swiftlet houses in Sarawak

Hazard identification (organism)	Health risk assessment			
	Main cause of the event			Risk of acquire resistance and pathogenicity event arising from main cause
	Antibiotic resistance	MAR index	Pathogenicity	
Bacillus amyloliquefaciens	Resistance to 1-2 types of antibiotic tested.	0.05-0.11	Previously reported as non-pathogenic (Priest et al., 1987)	Low
Bacillus botaviensis	Resistance to 6 types of antibiotic tested.	0.32*	Pathogenicity reported as unknown	Low
Bacillus cereus	Resistance to 8-10 types of antibiotic tested.	0.32*-0.47*	Enterotoxigenic genes (*hbla*, *entFM*) detected in most of the faecal and airborne isolates [+] Previously reported as volatile human pathogen (Bottone, 2010)	Low
Bacillus licheniformis	Resistance to 3 types of antibiotic tested.	0.16	Previously reported as food poisoning agent (Turnbull, 1996)	Low
Bacillus pumilus	Resistance to all types of antibiotic tested.	1.00*	Previously reported as pathogen to mango plant (Galal et al., 2006) and human (From et al., 2007)	Low
Bacillus subtilis	Resistance to 1-3 types of antibiotic tested.	0.05-0.16	Previously reported as food poisoning agent (Hoa et al., 2000)	Low
Bacillus thuringiensis	Resistance to 8 types of antibiotic tested.	0.37*	Insect pathogenic gene (*Bt*) detected (Argôlo-Filho & Loguercio, 2013)	Low
Bacillus spp.	Resistance to 1-8 types of antibiotic tested.	0.05-0.42*	Most of Bacillus species were harmless saprophytes (Hoa et al., 2000)	Low
Bacterium culalno	Resistance to 6 types of antibiotic tested.	0.32*	Pathogenicity reported as unknown	Low

Table 1 *(Continue)*

Hazard identification (organism)	Health risk assessment			
	Main cause of the event			Risk of acquire resistance and pathogenicity event arising from main cause
	Antibiotic resistance	MAR index	Pathogenicity	
Corynebacterium spp.	Resistance to 3 types of antibiotic tested.	0.16	Previously reported as non-pathogenic (Burkovski, 2008)	Medium
Enterococcus faecalis	Resistance to 1-6 types of antibiotic tested.	0.09-0.55*	Virulence gens (*ace*, *AS*, *efaA*, *gelE*) was detected +	High
Enterococcus harae	Resistance to 4-8 types of antibiotic tested.	0.09-0.36*	Depressed the young chickens growth and caused diseases in human in previous report (Poyart et al., 2002)	High
Enterococcus gallinarum	Resistance to 1-6 types of antibiotic tested.	0.09-0.55*	Previously reported as non-pathogenic (Gilmore et al., 2002)	High
Escherichia coli	Resistance to 1-10 types of antibiotic tested.	0.05-0.47*	No Shiga toxin (stx1 and stx2) was detected +	High
Klebsiella pneumonia	Resistance to 3 types of antibiotic tested.	0.16	Virulence gene (Gyr-B-2) detected in patients (Foysal et al., 2013)	High
Leucobacter iarius	Resistance to 5 types of antibiotic tested.	0.26*	Previously associated with pathogenic nematodes (Somvanshi et al., 2007)	Low
Lysinibacillus spp.	Resistance to 1-10 types of antibiotic tested.	0.05-0.53*	Previously reported as non-pathogenic	Low
Paenibacillus spp.	Resistance to 2-8 types of antibiotic tested.	0.16-0.42*	Pathogenicity reported as unknown (Ahmed et al., 2007)	Low
Pseudomonas aeruginosa	Resistance to 1 type of antibiotic tested.	0.11	Virulence gene (*oprL*) detected +	High
Sporosarcina aquimarina	Resistance to 2-7 types of antibiotic tested.	0.11-0.37*	Previously reported as non-pathogenic (Janarthine et al., 2012)	Low

Table 1 *(Continue)*

Hazard identification (organism)	Health risk assessment			
	Main cause of the event			Risk of acquire resistance and pathogenicity event arising from main cause
	Antibiotic resistance	MAR index	Pathogenicity	
Sporosarcina spp.	Resistance to 5 types of antibiotic tested.	0.42*	Previously report as non-pathogenic (Janarthine et al., 2012)	Low
Staphylococcus epidermitis	Resistance to 2 types of antibiotic tested.	0.11	Previously reported as non-pathogenic (Fey & Olson, 2010)	Medium
Staphylococcus kloosii	Resistance to 1-5 types of antibiotic tested.	0.05-0.26*	Previously reported as pathogen in human (Peer et al., 2011)	Medium
Staphylococcus lentus	Resistance to 1-5 type of antibiotic tested.	0.05-0.26*	Previously reported as non-pathogenic (Karachalios et al., 2006)	Medium
Staphylococcus nepalensis	Resistance to 2-8 types of antibiotic tested.	0.11-0.37*	Pathogenicity reported as unknown (Nováková et al., 2006)	Medium
Staphylococcus pulvereri	Resistance to 2 types of antibiotic tested.	0.11	Previously reported as pathogen to human and chicken (Zakrzewska-Czerwińska et al., 1995)	Medium
Staphylococcus sciuri	Resistance to 2 types of antibiotic tested.	0.11	Previously reported as highly pathogenic bacteria to the piglets (Chen et al., 2007)	Medium
Staphylococcus spp.	Resistance to 1-5 types of antibiotic tested.	0.05-0.26*	Previously reported as non-pathogenic (Madigan & Martinko, 2005)	Medium

Tested against: C: Chloramphenicol, Amp: Ampicillin, Te: Tetracycline, S: Streptomycin, CN: Gentamycin, E: Erythromycin, KF: Cephalothin, F: Nitrofurantoin, TOB: Tobramycin, RD: Rifampin, K: Kanamycin, SXT: Sulphamethoxazole/ Trimethoprim, AK: Amikacin, IPM: Imipenem, Cro: Ceftriaxone, P: Penicillin G, Do: Doxycycline, Caz: Ceftazidime, Nor: Norflaxacin, VA: Vancomycin, PRL: Pipercillin, CIP: Ciprofloxacin, NA: Nalidixic acid.

* MAR index ≥ 0.2 indicating a high risk of bacterial contamination and pose health hazard to human.
+ Pathogenicity indicated through multiplex-PCR in this study.

Table 2

Health risk assessment of the potential antibiotic resistant pathogenic airborne bacteria isolated from the swiftlet houses in Sarawak

Hazard identification (organism)	Health risk assessment			
	Main cause of the event			Risk of acquire resistance and pathogenicity event arising from main cause
	Antibiotic resistance	MAR index	Pathogenicity	
Bacillus cereus	Resistance to 9 types of antibiotic tested.	0.47*	Enterotoxigenic genes (*hbla*, *entFM*) detected in most of the isolates [+] Previously reported as volatile human pathogen (Bottone, 2010)	Low
Bacillus subtilis	Resistance to 1-3 types of antibiotic tested.	0.05-0.15	Previously reported as food poisoning agent (Turnbull, 1996)	Low
Bacillus spp.	Resistance to 2-7 types of antibiotic tested.	0.11-0.37*	Most of *Bacillus* species were harmless saprophytes (Hoa et al., 2000)	Low
Dermacoccus spp.	Resistance to 2 types of antibiotic tested.	0.11	Pathogenicity reported as unknown (David et al., 2012)	Medium
Enterococcus faecalis	Resistance to 1 type of antibiotic tested.	0.09	Virulence gens (*ace*, *AS*, *efaA*, *gelE*) detected [+]	High
Lysinibacillus fusiformis	Resistance to 5 types of antibiotic tested.	0.26*	Previously reported as unknown (Ahmed et al., 2007)	Low
Lysinibacillus spp.	Resistance to 2-4 types of antibiotic tested.	0.11-0.21*	Previously reported as non-pathogenic	Low
Microbacterium esteraromaticum	Resistance to 2 types of antibiotic tested.	0.11	Pathogenicity reported as unknown (Gneiding et al., 2008)	High
Paenibacillus taiwanensis	Resistance to 1 type of antibiotic tested.	0.05	Previously reported as unknown (Ahmed et al., 2007)	Low
Shingobacterium spp.	Resistance to 4 types of antibiotic tested.	0.21*	Pathogenicity reported as unknown	High

Table 2 *(Continue)*

Hazard identification (organism)	Health risk assessment			
	Main cause of the event			Risk of acquire resistance and pathogenicity event arising from main cause
	Antibiotic resistance	MAR index	Pathogenicity	
Sporosarcina aquimarina	Resistance to 2-19 types of antibiotic tested.	0.11-1.00*	Previously reported as non-pathogenic (Janarthine et al., 2012)	Low
Sporosarcina spp.	Resistance to 1 type of antibiotic tested.	0.05	Previously report as non-pathogenic. (Janarthine et al., 2012)	Low
Staphylococcus spp.	Resistance to 3 types of antibiotic tested.	0.16	Previously reported as non-pathogenic (Madigan & Martinko, 2005)	Medium
Staphylococcus kloosii	Resistance to 3 types of antibiotic tested.	0.16	Previously reported as pathogen in human (Peer et al., 2011)	Medium
Staphylococcus pulvereri	Resistance to 2 types of antibiotic tested.	0.11	Previously reported as pathogen to human and chicken (Zakrzewska-Czerwińska et al., 1995)	Medium

bacterial isolates. MAR index values higher than 0.2 are inferring that they have originated from high-risk sources where antibiotics are often used. MAR index values of less than or equal to 0.2 indicates a strain originated from sources where antibiotics are seldom or never used (Adeleke & Omafuvbe, 2011). This is an indication of a high presence of antibiotics selective pressure, which agrees with the report of Suresh et al. (2000). The bacteria were highly prevalent in developing a high MAR index mainly because of their tendency in accumulating multiple resistances under selection and antibiotic pressure (Berger-Bachi, 2002). This may imply an increase in antibiotic resistance with a higher risk of faecal and airborne bacterial contamination and potentially may pose threat to human health as antibiotic resistance decreases our ability in treating infections and diseases. The MAR index method is a good indicator in the differentiation of bacteria sources by applying antibiotics that are commonly used for human treatments (Osundiya et al., 2013). Tula and Iyoha (2014) discovered that antibiotic resistance increased with the increase of the MAR value. The bacterial isolates from swiftlet houses with the MAR index value of more than 0.2 were mainly from faecal origin. The multiple antibiotic resistance index of the bacterial isolates suggest that they have arisen from sources exposed to high level of antibiotics selective pressure resulting from non-specific, misuse or abuse of antibiotics. Kaneene et al. (2007) had reported that most of the *E. coli* with MAR index over 0.4 were isolated from faecal contaminated water surface. The increasing rate of MAR cases in various sites is spreading fast and poses threat to human health. The drug-resistant bacteria could also act as a reservoir of resistance plasmids which could be freely exchanged with possible pathogens in the intestine.

Indicator organisms, such as commensal *E. coli* and *Enterococcus faecium*, are of interest since they readily develop resistance to antimicrobials (Fair & Tor, 2014) and furthermore, they have the potential to disseminate their resistance genes to other bacteria, including pathogenic bacteria (Beceiro et al., 2013). Indicator bacteria could be transmitted to humans via the food chain in the same way as zoonotic food-borne bacteria, particularly since these bacteria will be present, potentially in high numbers, in most animal species. Hospital patients are especially at risk since *Enterococci* commonly causes hospital-acquired infections (Rice, 2001). The emergence of vancomycin-resistant *E. faecium* (VREf) is of concern since vancomycin is often used in hospitals to treat serious Gram positive bacterial infections (Garcia-Migura et al., 2005), and is chemically related to the growth promoter avoparcin, which was used in Europe since the 1970s until its ban (Acar et al., 2000).

In this study, most of the bacterial isolates showed a high level of antibiotics resistance to the antibiotics tested. This result is in agreement with Muhammad et al. (2010) who reported that the abuse and misuse of antimicrobial agents for growth promotion and prevention of diseases had impressed a

selective pressure that caused the discovery of more resistant bacteria. This is probably true with bacteria associated with faeces collected from swiftlet houses located in urban areas in this study. Hence, the antibiotic selection pressure for resistance by bacteria in birds is high and as a result, their faecal flora contains a high proportion of resistant bacteria. The results of this study on swiftlet indicted the possible biorisk in urban areas. Salehi and Bonab (2006) reported that the resistance of bacteria to the existing antimicrobial agents was widespread and of a great concern to poultry veterinarians. The use of antimicrobial in animal feed can also lead to the selection of antimicrobial resistant zoonotic enteric pathogens, which could be transferred to human through the consumption of contaminated food, or by direct animal contact.

Besides, *Enterococcus* spp. expressed with a high MAR index ≥ 0.2. The pathogenic antibiotic resistant *E. faecalis* isolated in a high frequency of occurrence or prevalence in the swiftlet houses may cause a health hazard to human because *E. faecalis* had been reported as a common pathogen causing infection in nosocomial (Olawale et al., 2011), surgical wounds, blood, urinary tract (Duprè et al., 2003). Although the occurrence of *Enterococcus gallinarum* was low in this study and reported as non-pathogenic by Gilmore et al. (2002), however, with a high MAR index indicating that *E. gallinarum* may become harmful to human. *Enterococcus* spp. has high gene transfer frequency and the gene may be exchanged between related or unrelated species, thus *Enterococcus* spp. may become hazardous to the environment and human health in the future if no prevention was taken.

According to Peer et al. (2011), *Staphylococcus kloosii* has developed linezolid resistance and caused intracranial bleed and sepsis in patients in India. Linezolid was a potent antimicrobial especially against Gram-positive microorganism such as methicillin-resistant coagulase-negative *Staphylococcus*, resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococci* and multidrug-resistant *Streptococcus pneumonia* (Diekema & Jones, 2001). Thus, *S. kloosii* may have the potency to pose a serious threat to humans in the future. *S. pulvereri* was normally isolated from human and diseased chicken carcass, thus it may act as a carrier for spreading the diseases (Zakrzewska-Czerwińska et al., 1995). Furthermore, According to Morita et al. (2014), *P. aeruginosa* has the ability to develop resistance to multiple antimicrobial agents and was able to mutate against antimicrobial agents thus it is considered as a high-risk pathogen. Hancock and Speert (2000) discovered that *P. aeruginosa* had higher intrinsic resistance than other bacteria to most recent antibiotics and decreased the clinical effectiveness. The health hazard existed for several human pathogens and the risk concerns not only the soil bacteria but also the bacteria that colonized various farm animals (*Enterococcus*, *Bacillus*) which are in contact with environmental bacteria (*Pseudomonas*). Consequently, the risk

may spread to food-borne pathogens which are frequently detected in animals and the dissemination of resistance genes may affect *Escherichia* and other bacterial species.

Exposure assessment predicts the outcomes if these antibiotic pathogenic bacteria are exposed (Table 3). The assessment identified various high-risk pathogen infections and high rate of antibiotic resistance transmission. This model believes that the overall risk of the occurrence and spread of the antibiotic resistance pathogen among humans is the highest. The risk of transmission from animal or even environmental reservoirs to humans is high. Infection may be mediated by the high risks of foodborne and waterborne transmission diseases caused by *Bacillus* spp., *Staphylococcus* sp., *Klebsiella* sp., *Enterococcus* sp., *Proteus* sp. and *Pseudomonas* sp. (Table 3). The assessment highlights the hazards, health risks and exposure of bacteria isolated in order to reduce the risk of the emergence and spread of antibiotic resistance. There are limitations in the assessment model because it only pays attention to pathogens of international concern. Besides, the exposure risks of each event stated in Table 3 cannot be generalised to pathogens not included here. This qualitative risk assessment provides early stages of development based on the main microbiological hazard determinant. A more systematic risk model needs to be constructed in the future which be possible to get accurate estimates of transmission risk and to quantitatively measure the effectiveness of interventions on defined outcomes.

Table 3

Exposure hazards assessment of these potential antibiotic resistant pathogen

Event	Exposure hazards on the likelihood of the event happening
Emergence in the human	Harmful to the human, immunodeficiency patients *Bacillus* sp. in the swiftlet faeces may cause food poisoning. *Staphylococcus* sp., *Bacillus* sp., *Klesiella* sp. and *Enterococcus* sp. isolates may cause diseases especially in human. *Proteus* sp. and *Pseudomonas* sp. may cause serious diseases in immunodeficiency patient. Over half of the bacterial isolates developed antibiotic resistance which may be a serious problem nowadays. High potential risk of health hazard to human.
Emergence in the plants and insects	Harmful mainly to the human and plants. Cause diseases among the insects. Some of the *Bacillus* sp. may affect the plant growth. Over half of the bacteria isolates developed antibiotic resistance which may be a serious problem nowadays. Medium potential risk to plants and insects.

Table 3 *(Continue)*

Event	Exposure hazards on the likelihood of the event happening
Emergence in the animals	Harmful to the animals. Lower the poultry production. *Enterococcus* sp. may slow down the poultry growth in farm. Over half of the bacteria isolates developed antibiotic resistance which may cause by illegal use of antibiotics as growth promoter. Food transmission to human may occur if contaminated meat ingested. High potential risk of health hazard to human.
Emergence in the environment	Harmful to the environment if these antibiotic resistance pathogenic gene transfer among the environmental bacteria. Contact with contaminated environment may cause potential health risks to the ecosystem. Medium potential risk to ecosystem.

CONCLUSION

Biorisk assessment of this study revealed that there is a possibility that the pathogenic bacteria may pose public health hazards in the future due to the presence of antibiotic residues in the environment and there is a probability that most pathogenic bacteria that threaten human health may soon be resistant to all known antibiotics. Interaction with human and agricultural waste materials may spread pathogenic bacteria including antibiotic resistant isolates to wildlife, potentially creating an additional environmental reservoir of antibiotic-resistant organisms.

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The Growth Potential and Bioaccumulation Ability of Probiotics under the Exposure of Different Heavy Metals

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ABSTRACT

The presence of heavy metals in aquaculture is a major concern due to possible toxicity effects to the organisms. Bioaccumulation with bacteria is an effective and economical way to remove heavy metals from the water. The objectives of this research were to measure the growth rate of probiotics (*Bacillus* sp. BpChIAY [BpChIAY] and *Bacillus thruingiensis*, [Bt]) under different concentrations of selected heavy metals, and to determine the ability of the probiotics to bioaccumulate selected metals. Bacterial strains were grown in nutrient broth with the addition of heavy metals (Cu, Cr, Cd, Zn, Ni) at 37°C to determine the growth under exposure to heavy metals. The bioaccumulation experiment was conducted by exposing the strains' pellets to heavy metals solutions. The concentrations of heavy metals were measured using Atomic Absorption Spectroscopy (AAS). The bacterial growth percentages when grown under 2 ppm Zn, Ni and Cd for BpChIAY were 128%, 103% and 67%, and for BT, the growth rates were 97.7%, 98.8% and 36.2%, respectively. The findings showed the order of the toxicity in the order from the most toxic: Cu>Cr>Cd>Zn>Ni. Both strains were able to bioaccumulate the heavy metals, and

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BpChIAY was a better metal absorber than BT at the rate of 0.0539 mg/g for Zn, 0.0781 mg/g for Ni and 0.0256 mg/g for Cd.

Keywords: *Bacillus* spp., *Chlorella* sp., heavy metals, probiotics, *Tor tambroides*

INTRODUCTION

The manufacturing industries, which consist of metal finishing processes such as electroplating, etching and preparations of metal components, are major contributors to heavy metals pollution in Malaysia. Among heavy metals released are nickel (Ni), zinc (Zn), aluminium (Al), cadmium (Cd), copper (Cu), iron (Fe) and chromium (Cr) (Yeoh, 1993). The metals concentrations in rivers of Malaysia have been extensively studied, which show that rivers sediments are mostly polluted with Pb, Zn and Cu due to industry discharges, sewage and runoff. Juru River in Penang, for example, contained 117 µg/g of Pb, 144 µg/g of Cu and 483 µg/g of Pb (Lim & Kiu, 1995). The Langat River of Negeri Sembilan contained 71-374 µg/g of Zn and 3.0-37.9 µg/g of Cd, which exceeded the natural average global shale values (Sarmani, 1989).

Heavy metals can have various toxicological effects on living organisms by disrupting the biochemical roles in metabolic processes such as the dysfunction of the endocrine system, reproductive system, growth, immune system as well as metabolism (Jakimska et al., 2011). This is an alarming issue because they can cause toxicity to aquatic organisms, being in close and prolonged contact with the soluble metals (Kaoud, 2015). Aquatic organisms may adsorb heavy metals from surrounding water and food, which may accumulate in various tissues and causing toxicological effects (Kaoud, 2015, Mazon et al., 2002).

Probiotics are known as living organisms that when applied in appropriate amounts will grant the hosts health benefits (Joint, 2002). They are able to provide benefits in fish where they can produce inhibitory responses against pathogens, provide essential nutrients as well as developing important immune responses (Verschuere et al., 2000). Some bacteria can reduce metal toxicity because they possess resistance mechanisms and are able to bind and sequester heavy metals onto the cell surfaces to remove heavy metals. *Lactobacillus* sp., for example, can reduce oxidative stress by heavy metals and detoxify them as well. For arsenic and mercury, *Lactobacillus* sp. resists by actively expulsing toxic metals from cytosol, govern by *mer* and *ars* operons in the DNA, respectively (Monachese et al., 2012). *Bacillus* spp., are Gram-positive, rod-shaped, and spore-forming bacteria. They are suitable to be consumed by living organisms due to their capability to withstand highly acidic conditions (Bader et al., 2012).

The concentration of heavy metals in the environment can be reduced by utilizing microorganisms through biosorption and bioaccumulation. The biosorption process refers to the binding of metals onto the cell wall's surface and it is a simple physicochemical process similar to conventional adsorption or ion exchange. Whereas bioaccumulation process refers to

the intracellular accumulation of metals that occur in two stages, biosorption and active transport system. This process is a much more complex process compared to biosorption in which it requires the metabolic activity of cells. In other words, the metals are required to go through biosorption first and only then the metals go through the next step, which is bioaccumulation by transporting them within the cells (Chojnacka, 2010).

Bioaccumulation works by transporting the metals across the cell wall and membrane. When the metals are within the cells, the metals will be bounded to intracellular structures. Intracellular accumulation and oxidation or reduction reactions are the mechanisms contributing to bioaccumulation in cells. There are several factors that impact the mechanisms which are the composition of growth medium, pH temperature, presence of other metals and inhibitors. The process of bioaccumulation relies on the synthesis of low molecular weight proteins metallothioneins that are rich with thiol groups that bind to the metals (Chojnacka, 2010).

Due to the hazardous effects of heavy metals to the environment, it is important to find appropriate ways to curb the issue of heavy metals pollution. Utilizing probiotics is an ideal method due to its metal bioaccumulation capabilities. Therefore, the objectives of this research are to measure the growth rate of the probiotics obtained from marine microalgae (BpChIAY) and freshwater fish (Bt) to grow under different concentrations of selected heavy metals, and to determine the ability of the probiotics to bioaccumulate the metals.

MATERIALS AND METHODS

Growth of Probiotics under Selected Heavy Metals

In this study, the following probiotics were used: *Bacillus* sp. BpChlAY isolated from marine microalgae, *Chlorella* sp., and *Bacillus thuringiensis* (Bt), isolated from freshwater fish, *Tor tambroides*. These strains were obtained from subculture from Faculty of Agriculture, UPM.

The probiotics were inoculated in 10 mL of nutrient broth in universal bottle for homogenizing purposes. The inoculated nutrient broths were placed on a rotary shaker at 160 rpm, for 24 hours at 37°C. After 24 hours, 50 µL of the homogenized probiotics were taken and placed in 5 mL of nutrient broth in HACH test tubes. The probiotics were grown for 8 hours on a rotary shaker at 160 rpm at 37°C. Absorbance readings for the probiotics were taken once every 2 hours until the final 8th hour. Experiments were done in triplicates. The readings obtained were then used to graph out growth curve graphs of the probiotics.

Probiotics Growth under Heavy Metals Analysis. The probiotics were homogenized in 10 mL of nutrient broth grown on a rotary shaker at 160 rpm, for 24 hours at 37°C. The selected heavy metals, Cd, Cr, Cu, Zn and Ni were added in 5 mL of nutrient broth in HACH test tubes and the probiotics were then inoculated in heavy metal containing nutrient broth. The absorbance readings for the probiotics were taken once every 3 hours until the final 6th hour. This experiment

was done only until the 6th hour because the bacteria's exponential phase started between the 2nd hour and the 6th hour. Experiments were done in duplicates. Two different concentrations of heavy metals (2 ppm and 10 ppm) were tested. The readings were then used to graph out the growth curve of the probiotics with heavy metals.

Bioaccumulation of Heavy Metals by Probiotics. 40 mL of bacterial culture, which were grown in nutrient broth, were centrifuged at 6000 rpm for 5 minutes. The pellets obtained were then washed with buffer and subsequently centrifuged. The supernatant was removed and the pallets in the falcon tubes were weighed. The pellets obtained were resolubilized and exposed to 50 mL of 2 ppm heavy metals solution. After two hours, the heavy metals solutions were extracted and sent to be analyzed under Atomic Absorption Spectroscopy (AAS) to identify the final concentration.

RESULTS AND DISCUSSION

As shown in Figure 1, BpChIAY underwent lag phase from 0th hour until the 2nd hour. The bacterium entered the exponential phase from the 2nd hour until the final 8th hour. The bacterium Bt showed a similar growth trend as BpChIAY in which the lag phase started from 0th hour to the 2nd hour, and entered the exponential phase from the 2nd hour until the 8th hour. There was no observable growth during the lag phase due to the bacteria synthesizing the necessary nutrients and molecules for growth, and to adapt to the new environment. In the exponential phase, the bacteria's population increased at the maximum rate. The exponential growth rates of Bt and BpChIAY were 0.09372hr^{-1} and 0.08292hr^{-1}, respectively.

Growth of the Probiotics under Selected Heavy Metals. It was observed that when grown under 10 ppm of heavy metals, none of the bacteria were able to grow (Table 1). When the concentration was reduced to 2 ppm, both bacteria showed positive growth (Table 1). For BpChlAY, the growth rate under Zn, Ni and Cd were 0.123 hr^{-1}, 0.123 hr^{-1} and 0.0848 hr^{-1}, respectively. Whereas for Bt, the growth rates were 0.106 hr^{-1} for Zn, 0.118 hr^{-1} for Ni and 0.0485 hr^{-1} for Cd.

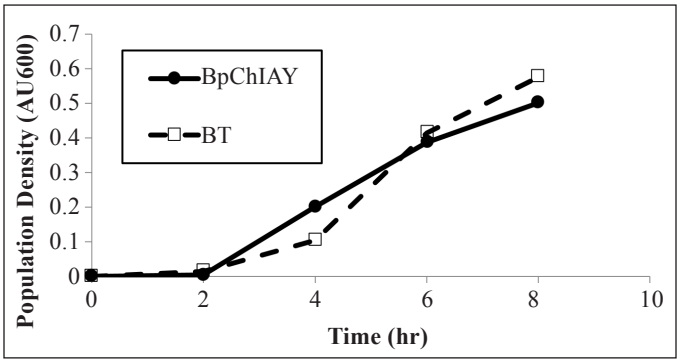

Figure 1. The growth of probiotics, BT and BpChIAY in nutrient broth

Table 1
The growth rates of BpChIAY *and* Bt *under different heavy metals*

	2 ppm		10ppm	
	BpChIAY growth rate (hr^{-1})	Bt growth rate (hr^{-1})	BpChIAY growth rate (hr^{-1})	Bt growth rate (hr^{-1})
Control	0.083	0.094	0.083	0.094
Cd	0.085	0.049	NA	NA
Cu	0.010	0.001	NA	NA
Cr	0.032	NA	NA	NA
Ni	0.123	0.118	NA	NA
Zn	0.123	0.106	NA	NA

NA = Not Available

BpChIAY growth rose higher when grown with Ni and Zn than the no-metal control at growth percentages of 128% and 103%, respectively (Figure 2). For Cd, Cu and Cr, BpChIAY's growth were inhibited where the percentages were only 67%, 5.02% and 22.2%, respectively (Figure 2). For Bt, only Cd, Ni and Zn did not inhibit growth whereas Cu and Cr inhibited it completely. Under Cd, Ni and Zn, the bacterium was able to grow at 36.2%, 98.8% and 97.7% and under Cu and Cr, the growth were completely inhibited (Figure 3).

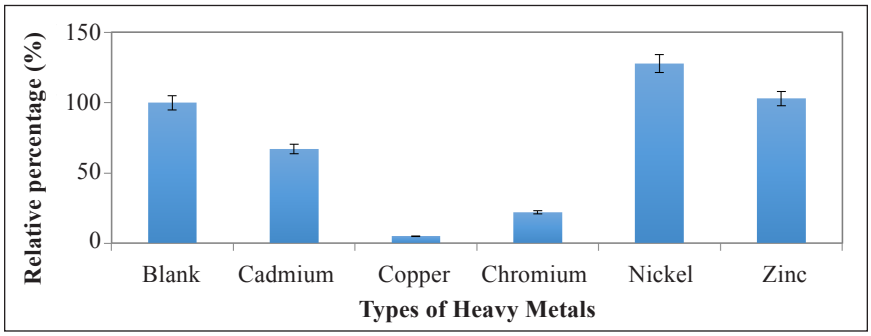

Figure 2. The percentage of growth of BpChIAY under different types of selected heavy metals

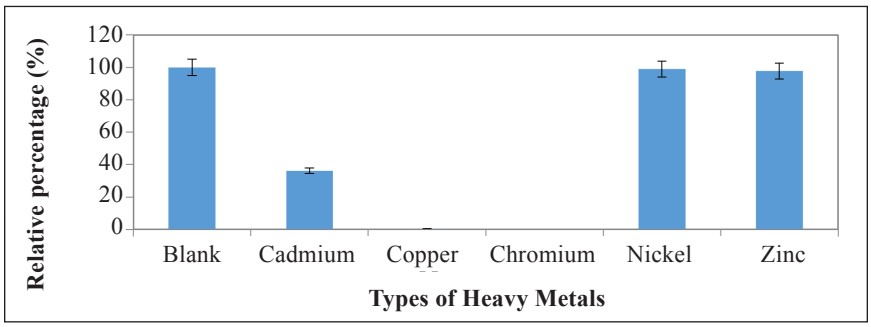

Figure 3. The percentage of growth of Bt under different types of selected heavy metals

The results showed that the bacteria were only able to grow when the concentration of heavy metals were reduced from 10 ppm to 2 ppm. This is expected because heavy metals at an increasing concentration can cause motility thus inhibiting the growth of the bacteria.

When bacteria are exposed to high concentrations of Zn, specifically Zn^{2+}, growth will be inhibited. This works by the reaction of Zn^{2+} with the mercapto group of the biological macromolecule which destroys the proteins and reject proliferation thus decreasing the rate of growth of cells. Besides that, Zn^{2+} can also cause the cell membrane permeability to change and disrupt the transportation of nutrients and waste across the membrane by deactivating the target sites in a cell (Yao et al., 2005).

For Cr, no growth were observed when the concentration was at 10 ppm or 2 ppm on both bacteria. This is because Cr, when presented in its toxic form, Cr^{6+}, may damage the cells' DNA. Cr^{6+} can easily be transported across the membranes due to non-specific phosphate or sulphate transporter activity. When Cr^{4+} is present within the cells, it will be reduced by reducing agents such as non-enzymatic Asc and thiol groups. The resulting Cr^{4+} intermediates will react and form complexes with the DNA, or proteins that will induce mutations, chromatid exchange and chromosomal instability (Younan et al., 2016).

Bacterial growth were also inhibited under the effect of Cu. Cu, when presented in high concentration interrupts the energy transport system, the enzyme active sites and the integrity of cell membranes similar to Zn. Cu can also cause the lipids, proteins and DNA within the bacteria to be damaged (Shenge et al., 2014). Similar to the other metals, the exposure of Cd interrupts the cell membrane functions and chemical reactions with cellular components. It can inhibit or compete with the cell's enzyme systems. This is caused by the interactions of the metals onto the cell surface receptors (Yamina et al., 2014).

Ni toxicity exhibits itself at 10 ppm to both of the bacteria. This is because there are four mechanisms of Ni toxicity on microorganisms which replaced essential metal of metalloproteins with Ni, the binding of Ni to catalytic residues of non-metalloenzymes, binding of Ni to the outside catalytic site of an enzyme to inhibit allosterically and lastly oxidative stress onto the microorganisms caused indirectly by nickel (Macomber & Hausinger, 2011).

BpChIAY and Bt can grow in Cd, Ni and Zn at low concentration due to their resistance mechanisms. There are three main mechanisms of action for bacteria against heavy metals which are ion exchange of the metals with peptidoglycan and teichoic acid, precipitation through nucleation reactions, and the complexation with oxygen and nitrogen ligands (Monachese et al., 2012).

The results also showed that the growth of BpChIAY grew significantly higher than the control when supplemented with 2 ppm of Zn by 28%. This is because Zn^{2+} in low concentrations, protect the integrated cells. This in return will cause the cells to have appropriate flow quality and is also helpful

in the synthesis of DNA and RNA (Yao et al., 2005). For Ni, trace concentrations of Ni can be utilized by microorganisms to assist in various cellular processes. One is by taking up Ni via nickel-specific permeases or ATP-binding binding systems after the microorganisms have sensed the Ni concentration. The metal will then be incorporated into enzymes that are nickel-dependent via complex assembly processes in which it will help the microorganisms to thrive in a Ni polluted environment (Mulrooney & Hausinger, 2003).

As obtained from the readings in Figures 2 and 3, the bacteria were able to thrive the best when supplemented with Ni and Zn, but growth was reduced by 33% for BpChIAY and 63.8% for Bt when grown under Cd. The order of toxicity of the metals to the bacteria was found to be Cu > Cr > Cd > Zn > Ni. This result was similar to a study done by Qing et al. (2007) that tested the growth of bacteria when grown under five different heavy metals. Qing et al. (2007) discovered that *Enterobacter clocae* and *Bacillus cereus* (*B. cereus*) are both tolerant to heavy metals particularly Cd and Zn. Malik et al. (2002) studied the metal tolerance of 70 industrial and agricultural soils bacterial isolates discovered that 88.8% of the bacteria isolates are tolerant to Ni, 82.8% to Zn and 71.4% to Cd.

Bioaccumulation of the Heavy Metals by Probiotics. BpChIAY metal absorbed per gram of bacteria for Zn was 0.0539 mg/g, 0.0781 mg/g for Ni and 0.0256 mg/g of Cd, whereas for Bt, the values for Zn, Ni and Cd were 0.0454 mg/g, 0.0309 mg/g and 0.0196 mg/g, respectively (Figure 4). When comparing the three metals, Zn and Ni have the higher removal rate compared to Cd for both bacterial strains. The ability of bacteria to adsorb heavy metals shows great usability in the open field like rivers in Malaysia. As an example, in Langat River in Selangor, the Zn concentration was observed to be at 71-374 ppm higher than the National Water Quality Standard of Malaysia, at 5 ppm (Sarmani, 1989).

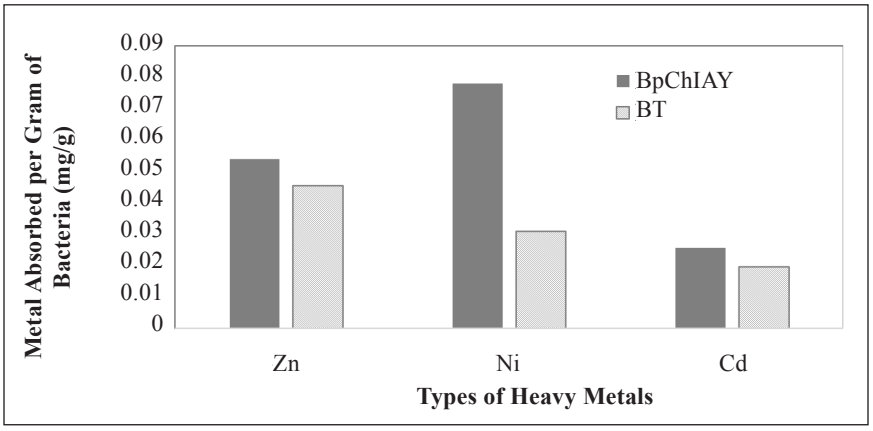

Figure 4. The average weight of heavy metal absorbed per gram of bacteria

The *Bacillu*s spp. are good metal adsorbers because they have higher adsorptive capacity from the high content of peptidoglycan and teichoic acid in the cell walls (Monachese et al., 2012). The carboxylic group in the glutamic acid of peptidoglycan of the bacteria's cell walls is a major site of metal deposition (Bhakta et al., 2012). For Cd-resistance bacteria, specifically gram-positive bacteria, its resistance depends on cadmium efflux mediated by the cadmium-exporting P-type ATPase CadA pump. An example would be in *Saccharomyces cerevisiae*, in which the Cd will be bounded by glutathione thus producing a Cd-bisglutathionato complex which will then be transported to the vacuole by an ABC transporter (Nies, 1999).

For the bioaccumulation of the heavy metals by the bacteria, only Cd, Ni and Zn at 2 ppm were selected to be tested with the bacteria as they did not inhibit growth entirely. It was observed that both bacteria are capable of accumulating Ni and Zn better than Cd. This confirmed previous results which showed that the bacteria grew better under Ni and Zn than Cd. When comparing the two bacteria's bioaccumulation ability, BpChIAY had a better rate of heavy metal removal from polluted environment compared to Bt. Ni was removed the most by BpChIAY followed by Zn and Cd. Bt removed Zn the most followed by Ni and Cd.

There are various ways of how microorganisms accumulate heavy metals through adsorptive interactions and metabolism mediated mechanisms. In metabolism mediated mechanisms, it is divided to metal transport within the cells for intracellular storage and intracellular detoxification (Juwarkar & Yadav, 2010). Intracellular detoxification is supported by the synthesis of metallothioneins (MT), proteins that are of low molecular weight. MT is produced by the cells when heavy metals are detected in the environment, and upon secretion, MT will bind to the heavy metals thus excluding it out of the cells' metabolical reactions (Chojnacka, 2010). The presence of MT in cells helps to increase the resistance against heavy metals and enhance the metal tolerance, sequestration or accumulation (Juwarkar & Yadav, 2010).

Costa & Duta (2001) studied the bioaccumulation capabilities of *Bacillus* spp., *B. cereus*, *Bacillus sphaericus* (*B. sphaericus)* and *Bacillus subtilis* (*B. subtilis*) which showed that the bacteria used were capable of bioaccumulating four types of heavy metals tested namely Cu, Zn, Cd and Pb with the best results shown by *B.subtilis* and *B.cereus*. Al-Taee (2015) studied the bioaccumulation ability of Bt isolated from soil, showed that the bacterium can grow in a Pb and Cd polluted environment. His results demonstrated that Bt was capable of accumulating Cd at 23.2 mg/g in the concentration of 100 mg/L. This is significant as the present study also tested the same bacterium capable of accumulating Cd.

CONCLUSION

The growth of both BpChIAY and Bt were observed under the effect of selected heavy metals at 2 ppm but no observable growth was obtained under 10 ppm. BpChIAY grew the best under Ni, Zn and Cd at growth percentages of 128%, 103% and 67% respectively. BT showed similar results as well by growing at 97.7% in Zn, 98.8% in Ni and 36.2% in Cd. However both bacteria showed little to no growth under Cu and Cr thus the order of toxicity of the heavy metals is in this order from the most toxic: Cu > Cr > Cd > Zn > Ni. This study also shows that the bacterial strains are capable of bioaccumulation where both bioaccumulated all three tested heavy metals, Cd, Ni and Zn. BpChIAY showed a higher metal absorbed per bacterial gram than Bt at 0.0539 mg/g for Zn, 0.0781 mg/g for Ni and 0.0256 mg/g for Cd**.** A recommendation in the future is to conduct more studies regarding on how to fully utilize these bacterial strains in the open field for the remediation of metals pollution in the environment. Other than the environment, the study on how we can use these strains in aquacultural species can be conducted to overcome heavy metal exposure to aquacultural species via ingestion as the *Bacillus* spp. are known to be able to grow under acidic environments.

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Effect of Growth Media Composition on Early Growth and Development of Moringa (*Moringa oleifera* L.) Seedlings

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ABSTRACT

In spite of huge economic and medicinal importance of moringa, its cultivation in Nigeria is still very low. This is largely due to the fact that sound agronomic practices have not been established. To do this, pot experiments were carried out at the Teaching and Research Farm, Ladoke Akintola University of Technology, Ogbomoso, Nigeria in 2015 to determine the effect of growth media composition on early growth and development of moringa seedlings. The growth media tested were 100% top soil, 100% compost, 100% sawdust, 75% soil + 25% compost, 50% soil + 50 % compost , 25% soil + 75% compost, 75% compost + 25% sawdust, 5% soil + 50% sawdust, 25% soil + 75% sawdust, 75% compost + 25% sawdust, 50% compost + 50% sawdust, 25% compost + 75% sawdust and 33.3% soil + 33.3% sawdust + 33.3% compost. The experiment was laid out in a Completely Randomized Design (CRD) replicated five times. Data collected, which included growth parameters such as seed germination (%), seedling vigour (scaled between 1 and 5), shoot weight, dry matter yield, stem height, stem girth and number of leaves and nutrient uptake, were subjected to analysis of variance and their means were compared using Duncan's Multiple Range Test (MRT) ($p<0.05$). Variation of growth media had significant effects on most of the parameter assessed. The result showed that 25% sawdust + 75% compost has the highest % seed germination 9 seedling/pot (92.6 %) and seedling vigour (4.5). Other growth media tested had less effect on the parameter studied. In addition, plants grown in 100% compost significantly outperformed others with regards to growth parameters' such as the stem height (91.66 cm), stem girth (1.95 cm), number of leaves/plant (14.53), fresh shoot (13.80 g), fresh root

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(28.36 g), dry shoot (5.90 g), dry root (7.50 g) and total dry matter yield (13.40 g). This superior performance is attributed to leaf nutrient uptake of 4.10, 0.56, 4.50, 1.40, 4.63, 87.47, 4.03 and 60.10 g/kg N, P, K, Ca, Mg, Fe, Cu, and Zn, respectively. Result showed that the use of 100% compost gave the best seedlings with the highest nutrient uptake. It is therefore concluded that the use of 100% compost produced high quality moringa seedling with optimum leaf nutrient uptake, hence it can be considered as an effective medium.

Keywords: Compost, growth media, nutrient uptake, sawdust, soil

INTRODUCTION

Moringa (*Moringa oleifera* L.), a member of Moringaceae family, is grown in the tropical and subtropical regions of the world. *Moringa oleifera* is a soft wood and native to India with great potential for its economical and medicinal (Adebayo et al., 2011; Sharma et al., 2011). It is commonly called drumstick tree and well known for its multi-purpose uses. It is widely adapted and regarded as the world most nutritious plant for human (Farooq et al., 2012) and for its culinary properties (Stevens et al., 2013). Moringa plants are known to have high amount of essential nutrients, vitamins, minerals and beta carotene (Gopalakrishnan et al., 2016). Apart from its medicinal uses, the plant leaves are good sources of amino acid (Okiki et al., 2015) and could be used as immune booster. All parts of moringa tree are useful and have long been used by human (Abdull et al., 2014). Moringa is a fast-growing, deep-rooted dicotyledonous plant with tuberous taproot system. It is drought-resistant and can thrive well in poor soils with little or no fertilization (Ndubuaku et al., 2014). Moringa plants are established either by cuttings or seeds (Mathenge, 2015). The seeds are either sown directly in the field or planted in nurseries (Ede et al., 2015) which subsequently influences their establishment and productivity in the field.

The juice extracted from the leaves can be used to make foliar nutrient capable of increasing crop yield (Matthew, 2016; Merwad, 2017). The leaves also provide excellent materials for the production of biogas (Kivevele et al., 2011). It is rich in health promoting photochemical such as carronades, phenolics (chlorogenic acid), flavonoids (quercelin and kaempherol glycoside), various vitamins and minerals (Okiki et al., 2015; Udikala et al., 2017). *Moringa oleifera* is often grown as fence plant (Amaglo et al., 2007).

Growth media play an important role in seed germination, seedling growth and vigour. Potting media assist in the regulation of growth and the development of seedlings produced (Bhardwaj, 2014; Popescu & Popescu, 2015) which subsequently influence their establishment and productivity in the field. In Nigeria, the traditional method of potting medium is topsoil dug from farmland and amended with manure. This could render the land unproductive for cropping which could also be prone to erosion and degradation (Baumhardt et al., 2015; Pimentel & Burgess, 2013). The quality of media composition used could

influence the seedling obtained (Bhardwaj, 2014; Desai et al., 2017; Tian et al., 2017).

Baiyeri and Mbah (2006) reported the relative importance of soilless media for growing potted ornamental plants in Nigeria. Percentage germination, seedling emergence and growth in different sowing media were affected by the physical and chemical compositions of the growing media. The use of coarse materials as growth media ensured greater aeration and drainage of the media and also enhanced germination and seedling emergence (Baiyeri & Aba, 2007).

Despite the economic and medicinal importance of this crop, its cultivation still remains low. In order to encourage its large scale cultivation, there is a need to develop sound agronomic practices, hence the need for the present study. Therefore, this paper reports the effects of growth media composition on early growth and development of moringa (*Moringa oleifera*) seedlings.

MATERIALS AND METHODS

The Experimental Site

The experiments were carried out in February-May and August-November 2015 at Teaching and Research farm of Ladoke Akintola University of Technology, Ogbomoso, Nigeria. Ogbomoso, is located on latitude 8°10'N of the equator and longitude 4°16'E. It is located in the Guinea Savanna Zone of Southwest Nigeria. The temperature of the area ranges from 28°C to 33°C with humidity of about 75 % all year except in January when the dry wind blows from the North. Rainfall distribution is bimodal and extends for eight to nine months of the year. On the average, the total annual rainfall is about 128 mm. The soil of the site belongs to the USDA classification of Alfisol, which is moderately drain, ferruginous tropical soil with a sandy loamy texture. The vegetation covers of the site are characterized by scattered trees and shrubs and by cynodon species.

Soil Analysis

Soil samples analyses was done for physical and chemical properties. Soil samples were collected at the depth of 0 – 15 cm. The sample was air-dried, crushed and sieved for the determination of pH, bulk density, total nitrogen (N), available phosphorus (P), exchangeable potassium (K) , calcium (Ca), magnesium (Mg), ferrum (Fe), copper (Cu), zinc (Zn) and manganese (Mn) and carbon (C).

Experimental Materials

The growing media and the dosage rate used for this study were: 100% soil (S), 100% Sawdust (SD), 100% compost (C), 75% soil + 25% compost, 50% soil + 50% compost, 25% soil + 75% compost, 75% soil + 25% Sawdust, 50% soil + 50% sawdust, 25% soil + 75% sawdust, 75% compost and 25% sawdust, 50% compost + 50% sawdust, 25% compost + 75% sawdust, 33.3% soil + 33.3% sawdust + 33.3% compost and PK 1 moringa seeds.

Experimental Design and Management

The pot experiment consisted of 65 pots. Each treatment had 5 pots considered as a replication laid out in complete randomized design. The potting media were perforated at the base to enhance aeration and drainage. Each pot was filled with 5 kg of its respective treatment combination and dosage. At planting, ten moringa seeds (PK 1 variety from India) were sown in the potted media at depth of 1.5 cm. The pots were watered heavily immediately after sowing and subsequently once on alternative days. Weeding was done by hand roughing of the weeds as they emerged. Physical and chemical properties of the sowing media were determined through laboratory analyses before sowing the seeds.

Data Collection

Percentage seeding germination, that is, seedling emergence was recorded when the first foliage leaf appeared using the method described by Ede et al. (2015):

$$\% SG = \frac{N_{gs}}{T_{nsp}} \times 100 \quad (1)$$

where SG - Seeds Germination; N_{gs} -Number of germinated seeds; T_{nsp} -Total number of seeds planted. The plant height was measured from the root level to the tip of the shoot using tape rule. The stem girth was determined by taken the circumference of the stem and multiplying the value by $\frac{22}{7}(\pi)$ (Ede et al., 2015):

$$\text{Stem girth} = \text{Stem diameter} \times \frac{22}{7}(\pi) \quad (2)$$

The number of leaves was obtained by counting the number of fully expanded leaves per seedlings. Dry matter yield: Three plants per plot were uprooted (destructive sampling) at harvesting, the plants were separated into different parts (stem, root, and leaf), cut into pieces, bagged in separate brown envelope and oven dried to constant weight at 80°C. From these, dry weights of each plant parts were obtained.

Plant tissue nutrient contents and uptake: For determination of the leaf N, P, K, Ca, Mg, Fe, Cu, and Zn contents, three fully expanded leaves were selected per plant. Sampled leaves were oven dried at 80°C for 72 h to the constant weight and grounded in a Willey mill to reduce the material to a fineness suitable size. The grounded samples were stored in airtight plastic containers for chemical analysis. Total nitrogen was determined by digesting 0.5 g dry leaf samples with 68% H_2SO_4 in Kjeldahl digestion unit until sample colorless and titrated with 0.1 N of H_2SO_4 using selenium and sodium as catalyst. Total N was determined from the digest by stem distillation with excess NaOH. The P, K, Ca, Mg, Fe, Cu and Zn plant tissue contents was determined by ashing 0.2 g of the plant samples in muffle furnace at 600°C for 3 hours. The ash was cooled and dissolved in 1N hydrochloric acid and the solution passed through filter paper into 50 ml volumetric flask and was made up to the mark with distilled water. From the digest, P concentration was determined by the vanadomolybdate yellow colorimetry method using spectrophotometer. The K

was determined by using flame photometer (Cornin Model 400) while micronutrient (Fe, Cu, Zn) were estimated with atomic absorption spectrophotoneter (Perken Elmer AAS-300). Nutrient accumulation in plant was evaluated using the method used by Akanbi et al. (2002) as:

Nutrient uptake =
% Tissue nutrient content
× sample dry weight (3)

Data Analysis

Data collected over the two trials were pooled before subjected to statistical analysis using standard analysis of variance (SAS 2000) for complete randomized design. The significance of the treatment effect was determined using the F-test and mean separation was done with Duncan's Multiple Range Test (MRT) at 5% probability level.

RESULTS

The physical and nutrient analysis of the growing media was determined and the result is presented in Table 1. The highest pH (7.6) and least bulk density (0.09) were obtained in 100% compost while the least pH (6.3) and highest bulk density (1.4) were obtained from 100% soil. Among the growth media, 25% S + 75% C had highest total Nitrogen (1.11) while the least was 100% SD (0.60) and 25% S + 75% SD (0.60) (Table1). The 100% compost had the highest P and K (0.97 and 3.90, respectively) while the least were from 100% soil (0.00) followed by 75% C+ 25% SD (0.30). The 25% C + 75% SD had the highest Ca (5.70) while the least was from 100% SD (0.93). Highest Mg was recorded from 25% S + 75% SD and 33% S + 33% C + 33% SD (0.51) while the least came from 100% soil (0.07). The growth media 50% C and 50% SD had the highest Fe (0.71) while least Fe (0.09) was obtained from 100% SD. The 100% SD had the highest Cu (64.00) while least Cu (21.00) came from 100% soil. The 25% S + 75% SD had the highest Zn, C, C: N, (340.33, 40.70 and 68.33) while the least Zn (78.33), C (14.20) and C: N (13.00) from 100% soil and 100 % SD.

Effect of Growth Media on Seed Germination (%)

Figure 1 shows that the effects of growth media composition on germination (%) and seedling vigour are significant at ($p<0.05$). The treatment 25% S + 75% C has the highest germination rate (92.6) while 100% SD has the least (51) (Figure 1a). There was no significant difference in seedling vigour of growth media of 100% compost and 25% S + 75% C (Figure 1b). These two growth media have the same seedling vigour rating of (4.5) while treatment of 25% C + 75% SD had the least (2.80).

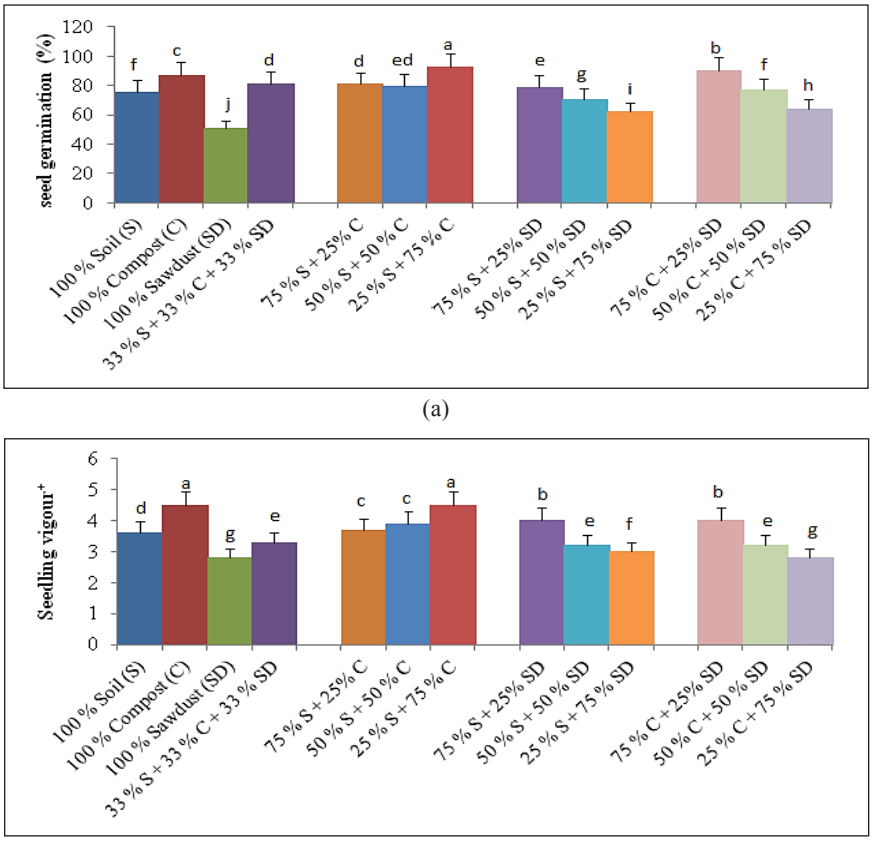
Effects of Growth Media Composition on Growth Parameters of Moringa Seedlings

Response of moringa seedlings to different growth media composition are presented in Figures 2 and 3. Growth media compositions had significant ($p<0.05$) effect on stem height, stem girth and number of leaves at different growth stages.

Table 1
Chemical composition of different growth media used for the experiment

Media Growth Composition	pH	Bulk Density	N	P	K	Ca	Mg	Fe	Cu	Zn	C	C: N
		g/cm	g/kg					mg/kg				
100% Soil (S)	6.3	1.4	0.63^{e}	0.25^{h}	0.54^{i}	1.20^{g}	0.07	0.12	21.00^{j}	78.33	14.20^{m}	22.33^{i}
100% Compost (C)	7.6	0.09	0.60^{ef}	0.97^{a}	3.90^{a}	0.93^{g}	0.46	0.32	64.00^{a}	332.33	21.43^{i}	13.00^{k}
100% Sawdust (SD)	6.6	0.9	0.62^{e}	0.72^{b}	1.12^{h}	5.30^{ab}	0.41	0.09	34.63^{e}	316.00	39.33^{b}	62.00^{b}
75% S + 25% C	6.7	1.26	0.72^{c}	0.55^{e}	1.65^{ed}	3.20^{e}	0.21	0.31	27.10^{i}	148.33	16.13^{l}	22.33^{i}
50% S + 50% C	6.9	1.22	0.78^{bc}	0.64^{d}	1.62^{e}	3.00^{e}	0.24	0.37	32.03^{gh}	162.66	18.10^{k}	22.66^{i}
25% S + 75% C	7.1	0.19	1.11^{a}	0.72^{b}	1.71^{d}	3.80^{d}	0.22	0.30	47.10^{c}	171.33	20.13^{j}	41.00^{f}
75% S + 25% SD	6.7	1.35	0.80^{b}	0.31^{g}	1.31^{g}	3.96^{d}	0.42	0.20	30.13^{h}	310.33	30.53^{e}	38.00^{g}
50% S + 50% SD	6.8	1.31	0.82^{b}	0.41^{f}	1.42^{f}	4.80^{bc}	0.45	0.20	41.06^{e}	302.33	33.56^{c}	45.33^{e}
25% S + 75% SD	7.0	1.08	0.60^{ef}	0.69^{bc}	1.60^{e}	5.03^{bc}	0.51	0.22	33.63^{fg}	340.33	40.70^{a}	68.33^{a}
75% C + 25% SD	7.2	0.15	0.62^{e}	0.71^{b}	0.30^{j}	4.80^{bc}	0.42	0.62	60.10^{a}	280.33	29.30^{f}	55.33^{c}
50% C + 50% SD	7.1	0.18	0.62^{e}	0.41^{e}	0.31^{j}	$4.^{70c}$	0.41	0.71	54.33^{d}	314	28.03^{g}	45.33^{e}
25% C + 75% SD	7.1	0.21	0.64^{de}	0.65^{ed}	1.60^{e}	5.70^{a}	0.50	0.62	60.10^{a}	278.33	31.43^{d}	48.33^{d}
33% S + 33% C + 33% SD	7.0	0.23	0.71^{cd}	0.73^{b}	2.64^{b}	2.40^{f}	0.51	0.55	33.20^{fg}	270.33	25.56^{h}	35.33^{h}

Note. Means along the column with the same letter are not significant at $p \leq 0.05$



(a)

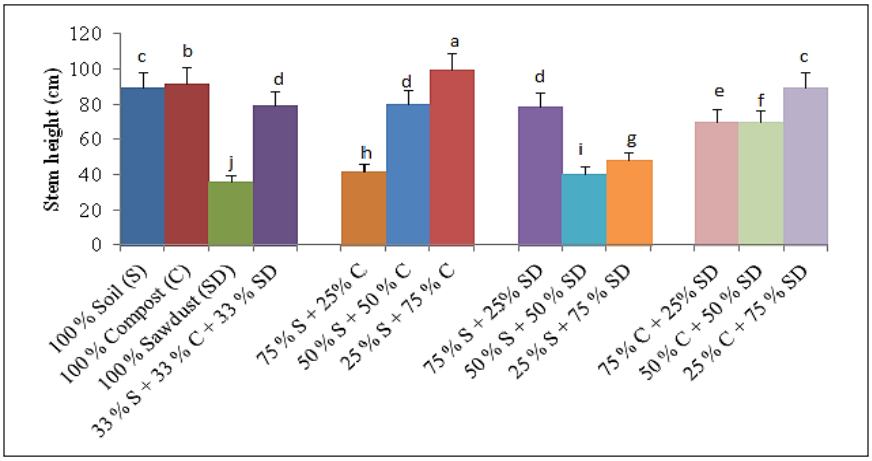
(b)

Figure 1. (a) Effects of growth media composition on percentage seed germination; and (b) seed vigour of moringa seedling
Note: + Scaling rating 1-5, where 1=Very poor and 5= Excellent

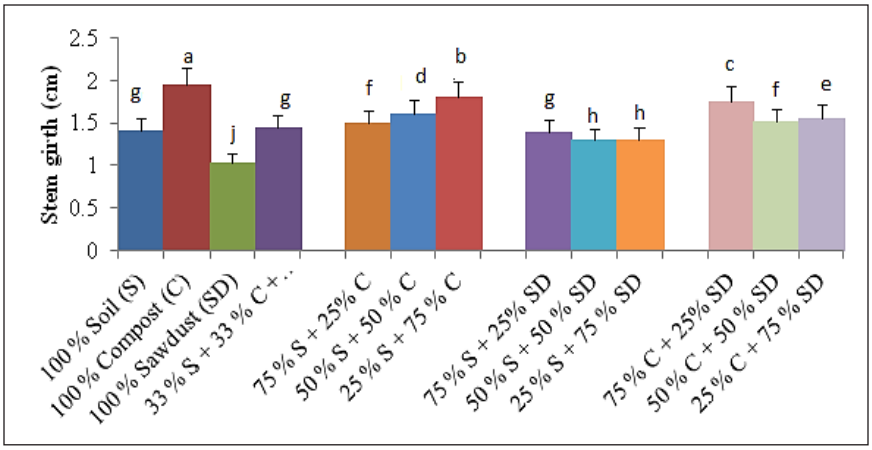
Figure 2a showed the effects of growth media on stem height. At 12 WAS, application of 25% S + 75% C growth media composition had the tallest plant (99.33 cm/plant) while 100% sawdust had the shortest plant. Among the pure substrates and their combination, 100% compost had the tallest plant (91.66 cm/plant) while 100% sawdust had the shortest plant (36.33 cm/plant). In addition, for different combination level of soil and compost, as the level of soil decreases and increases in compost (25% S + 75% C) performed better (99.33 cm). Again, when different levels of soil were mixed with sawdust, the higher the level of soil and lower the sawdust (75% S + 25% SD) gave better stem height (79.00 cm). From the combination of different level of compost and sawdust, 25% C + 75% SD had the longest stem (89.33 cm/plant). For stem girt (Figure 2b), the moringa seedling grown in 100% compost had the robust stem girth at 12 WAS with the values of 1.95 cm/plant followed by mixture of 25% S + 75% C (1.8

cm/plant) while the least was recorded with the growth media composition of 100% SD (1.04 cm/plant).

For number of leaves at these stages (Figure 3), the value of 14.53 cm were recorded for seedling grown with 100 % compost at 12 WAS while 25% C + 75% SD had the least number of leaves (10.00/ plant). Among the pure substrates and their combination, 100% compost had the highest number of leaves/plant (14.53). In addition, for different combination level of soil and compost, as the level of soil decreases and increases in compost (25% S + 75% C) gave better number of leaves/plant (12.40). Again, when different levels of soil were mixed with sawdust, combination of 50% S + 50% SD and 25% S + 75% SD gave the same number of leaves/plant (11.2). From the combination of different level of compost and sawdust, 50% C + 50% SD had the highest number of leaves/plant (12.73).



(a)



(b)

Figure 2. (a) Effects of growth media composition on stem height; and (b) and stem girt of moringa seedling

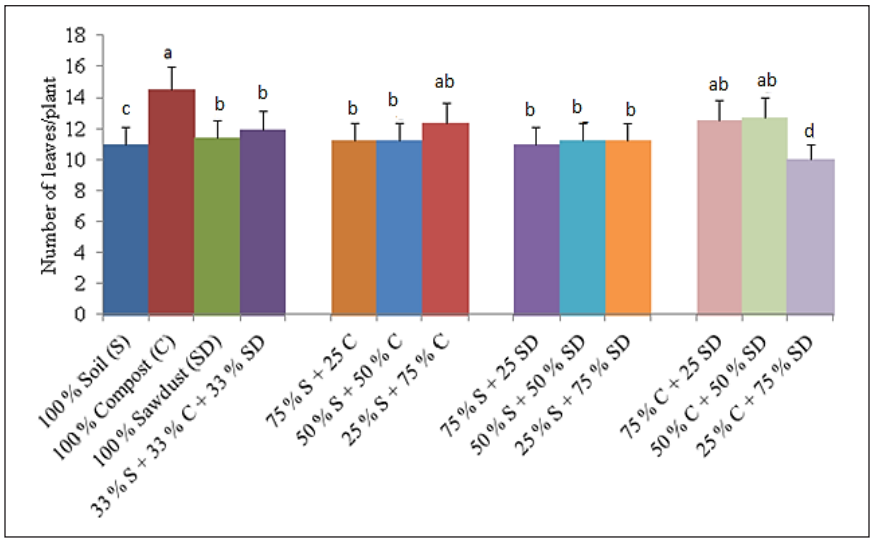


Figure 3. Effects of growth media composition on number of leaves/plant of moringa seedlings

Effects of Growth Media Composition on Fresh and Dry Matter Weight

Growth media composition has significant ($p<0.05$) effect on fresh and dry matter weights of moringa seedling at 12 weeks after sowing (Table 2). Grow media of 100% SD has the highest fresh shoot weight (34.63 g) followed by 25% S + 75% SD and 25% C + 75% SD with the values of 33.63 g and 33.20 g, respectively while the least (11.40 g) was recorded with growth media composition of 75% C + 25% SD. Dry shoot weight, dry root weight and total dry matter weight of seedlings differ significantly ($p<0.05$) at 12 WAS with different growth media composition. High fresh root, dry shoot weight and root weight and total dry matter weight were the highest with growth media composition of 100% compost (28.36 g, 5.90 g, 7.50 g, and 13.40 g respectively) while 100 % sawdust has the least fresh root weight, dry shoot weight and dry root weight with the values of 19.00 g, 3.96 g, 2.96 g and 6.29 g, respectively. Figures S1 – S2 reveal the correlations between total dry matter yield and nutrient uptake while the correlations between the total dry matter and media available nutrients were presented in Figures S3 – S4. The R^2 values of the nutrient uptakes ranged from 0.0158 to 0.827 while 37.5% had the positive strong correlation on nutrients uptake. For the media available nutrients, the R^2 values ranged from 0.0001 to 0.3239. Generally, there is week correlation between the dry matter yield and media available nutrients.

Table 3 showed the effects of media composition on nutrient uptake moringa plant. The leaf N, P, K, Ca, Mg, Fe, Cu and Zn uptake were significantly affected by media composition. For N, P, K, Ca, Mg, Fe, Cu and Zn, the uptakes were highest with 100% compost while the least for all the elemental composition varies among the other media composition.

Table 2
Effects of growth media composition on fresh and dry weights of different parts of moringa seedlings at 12 WAS

Growth Media Composition	Fresh Shoot Weight (g)	Fresh Root Weight (g)	Dry Shoot Weight (g)	Dry Root Weight (g)	Total Dry Matter Weight (g)
100% Soil (S)	21.00j	24.06c	4.02c	5.03d	9.05^{h}
100% Compost (C)	13.80^{a}	28.36^{a}	5.90^{a}	7.50^{a}	13.40^{a}
100% Sawdust (SD)	34.63^{f}	19.00^{g}	3.96^{c}	2.96^{c}	6.92^{j}
75% S + 25% C	12.00^{bc}	24.56^{c}	4.03^{c}	6.90^{c}	10.93^{e}
50% S + 50% C	32.03^{gh}	24.63c	4.03^{c}	7.03^{a}	11.06^{c}
25% S + 75% C	12.40^{b}	26.06^{b}	5.80^{a}	7.00^{ab}	12.80^{b}
75% S + 25% SD	30.13^{h}	24.60^{c}	4.20^{c}	4.90^{d}	9.10^{g}
50% S + 50% SD	12.40^{d}	21.63^{e}	3.96^{c}	5.00^{d}	8.96^{i}
25% S + 75% SD	33.63f^{g}	20.03^{f}	5.00^{b}	4.93^{d}	9.93^{f}
75% C + 25% SD	11.40^{c}	24.60^{c}	5.00^{b}	6.00^{c}	11.00^{d}
50% C + 50% SD	12.40^{b}	24.53^{c}	4.93^{b}	6.20^{bc}	11.13^{c}
25% C + 75% SD	33.63^{fg}	24.10^{c}	5.00^{b}	6.00^{c}	11.00^{d}
33% S + 33% C + 33% SD	33.20^{fg}	24.70^{c}	4.96^{b}	5.96^{c}	10.92^{e}

Note: Means along the column with the same letter(s) are not significantly different at $p \leq 0.05$

Table 3
Effects of growth media composition on nutrient uptake of moringa seedlings

Growth Media Composition	N	P	K	Ca	Mg	Fe	Cu	Zn
	---------- g/kg ----------					---------- mg/kg ----------		
100% Soil (S)	2.47^{c}	0.36^{c}	3.10^{d}	0.72^{e}	0.31^{h}	52.47^{j}	3.67^{ab}	24.53^{i}
100% Compost (C)	4.10^{a}	0.56^{a}	4.50^{a}	1.40^{a}	4.63^{a}	87.47^{a}	4.03^{a}	60.10^{a}
100% Sawdust (SD)	2.40^{c}	0.50^{ab}	2.20^{e}	0.84^{de}	0.63^{b}	60.47^{g}	2.86^{d}	38.13^{h}
75% S + 25% C	2.97^{b}	0.40^{c}	3.23^{d}	1.17^{bc}	0.33^{gh}	50.57^{l}	3.90^{ab}	51.33^{c}
50% S + 50% C	3.00^{b}	0.31^{c}	3.96^{bc}	1.00^{cd}	0.51^{c}	53.33^{h}	3.67^{ab}	49.33^{e}
25% S + 75% C	3.28^{b}	0.43^{bc}	4.20^{ab}	1.32^{ab}	0.57^{b}	7.37^{b}	3.60^{ab}	58.20^{b}
75% S + 25% SD	1.99^{c}	0.38^{c}	3.30^{d}	0.70^{e}	0.37^{fg}	51.47^{k}	3.40^{bc}	38.16^{h}
50% S + 50% SD	2.97^{b}	0.30^{c}	3.00^{d}	0.81d^{e}	0.41^{ef}	53.97^{i}	3.00^{cd}	50.23^{d}
25% S + 75% SD	2.40^{c}	0.32^{c}	3.20^{d}	0.74^{e}	0.49^{c}	50.47^{l}	2.97^{cd}	51.57^{c}
75% C + 25% SD	3.00^{b}	0.39^{c}	4.00^{ab}	1.20^{abc}	0.40^{ef}	72.10^{d}	3.93^{a}	57.47^{b}
50% C + 50% SD	2.97^{b}	0.37^{c}	3.93^{bc}	1.10^{bc}	0.41^{ef}	68.03^{f}	3.40^{bc}	42.57^{g}
25% C + 75% SD	3.20^{b}	0.32^{c}	3.20^{d}	1.12^{bc}	0.43^{e}	71.10^{e}	3.60^{ab}	44.00^{f}
33% S + 33% C + 33% SD	3.21^{b}	0.37^{c}	3.40^{cd}	1.13^{bc}	0.44d^{e}	73.47^{c}	3.63^{ab}	43.97^{f}

Note: Means along the column with the same letter are not significant at $p \leq 0.05$

DISCUSSION

Majority of the parameters for moringa such as seedling germination, seedling vigour, stem height, stem girth, number of leaves, fresh shoot weight, fresh root weight, dry shoot weight, dry root weight and total dry matter yield distinctively differ with the growth media composition. Growth media with adequate aeration and moisture content improve seedling germination (Atiyeh et al., 2000; Cáceres et al., 2015; Noorhosseini et al., 2018).The differences in the organic components in the different growth media composition could be the possible cause of differential growth performance. Better vegetative growth was recorded with seedlings treated with compost. This could be due to the fact that compost has the highest pH, lower bulk density, N, P and K which could enhance vegetative growth of the moringa seedlings. Higher nutrient uptake in compost is responsible for promoting better seedling vegetative growth and provides high water-holding capacity, good aeration and stimulates warmth which facilitates germination. This is supported by the reports of Baloch et al. (2014) and Castellanos et al. (2011).

The highest fresh shoot weight and total dry matter yield was recorded by 100 % compost could be due to high uptake of N, P, and K and Ca by the plant which is responsible for holding together the cell walls of plants as new tissue such as root tips, young leaves and sloop tips often exhibit distorted growth from improper cell wall formation when they are nutrient deficient (Hao & Papadopoulos, 2004). The texture or particle size distribution of nursery soils and that of potting medium for containerized planting stock is an important soil physical property influencing root and shoot growth (Dolor, 2011). This result is also in line with the findings of Goss et al. (2013) that high calcium content increases total biomass yield, fruit dry matter and fruit yield of tomato plants. The result of Domingues et al. (2016) also shows that high calcium concentrations in the growth media lead to higher dry mass of the shoot and root, high grain yield and high calcium concentration in the leaves and grains.

Better stem girth value was recorded with 100 % compost growth media was due to fact that the amount of nutrient uptake and availability by the seedlings in this growth medium is higher than other growth media. As a result of this, the nutrients were readily made available and used. This result is similar to the findings of Asante et al. (2012) which posited that compost had the highest stem height and stem girth. This indicates that stem height growth goes with the growth in stem girth.

Different growth media compositions have significant effect on number of leaves/ plant. The use of 100% compost gave the highest number of leaves/plant over other growth media. This result confirms the findings by Peter-Onoh et al. (2014). The best soil for growing vegetables is one that is well drained, and has high amount of nutrient and organic matter content. Similar trends were observed with the uptake of the nutrients by the seedlings. And in most cases seedlings performance were similar where

100% compost or 75% compost + 25% soil were used. This is also in line with the findings that sand and compost combination gave best result for growth and survival of seedlings in the nursery (Bali et al., 2013; Heidari & Mohammad, 2012; Radin et al., 2017).

CONCLUSION

The use of different growth media had significant effects on most of the parameters assessed on moringa seedlings. Generally, percentage germination rate is high for all the growth media. The use of compost only (100% compost) or compost in combination with soil (75% compost + 25% soil) gave the best moringa seedling with the highest number of leaves/plant, fresh root weight, dry shoot weight, dry root weight, % seed germination and nutrient uptake. It was concluded that the use of 100 % compost as growth medium could be the best for production of high quality moringa seedlings.

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APPENDIX

Figures 1 and 2 showed correlation between total dry matter yield and nutrient uptake. There is positive correction between total biomass yield and nutrient uptake.

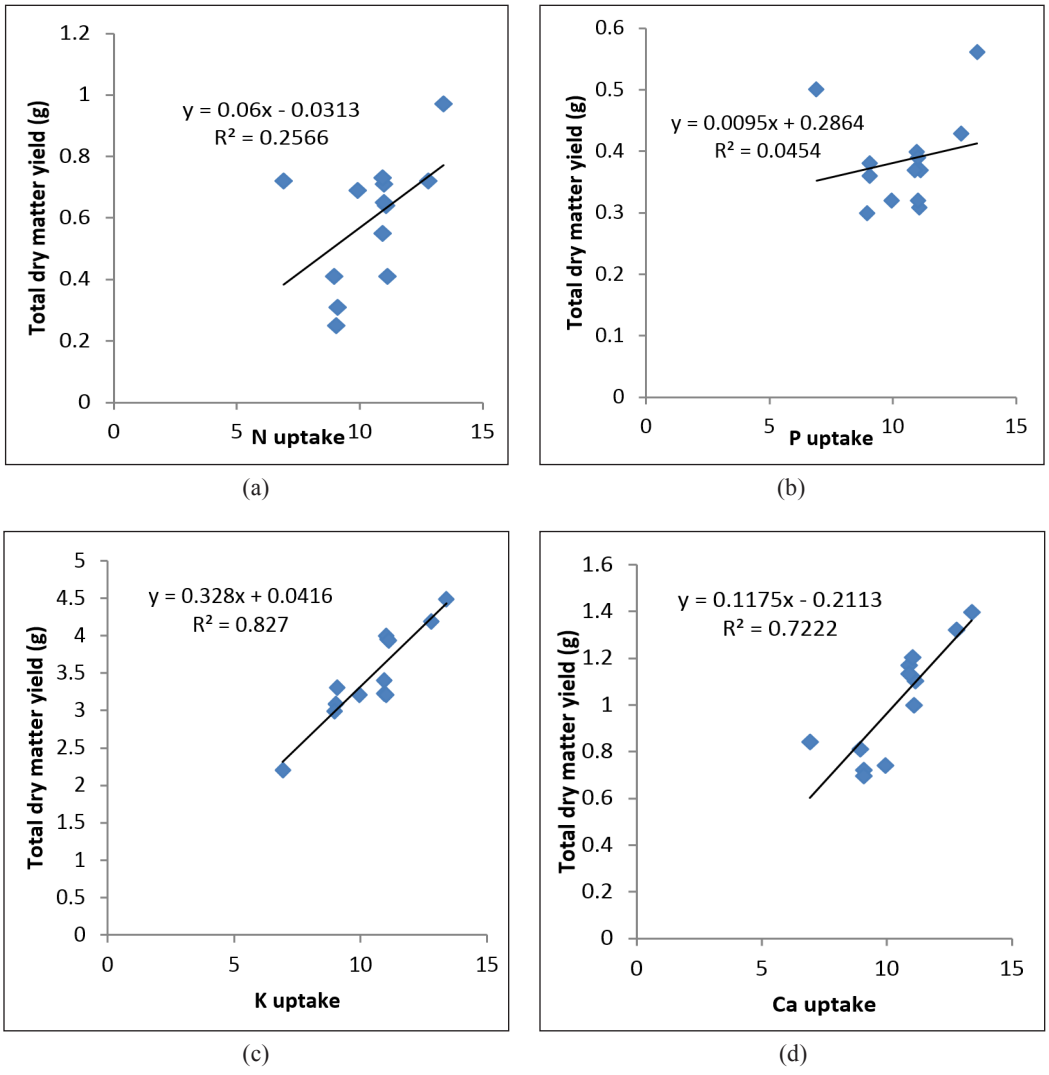

Figure S1. Correlation between total dry matter yield and nutrient uptake of (a) N ; (b) P; (c) K; and (d) Ca

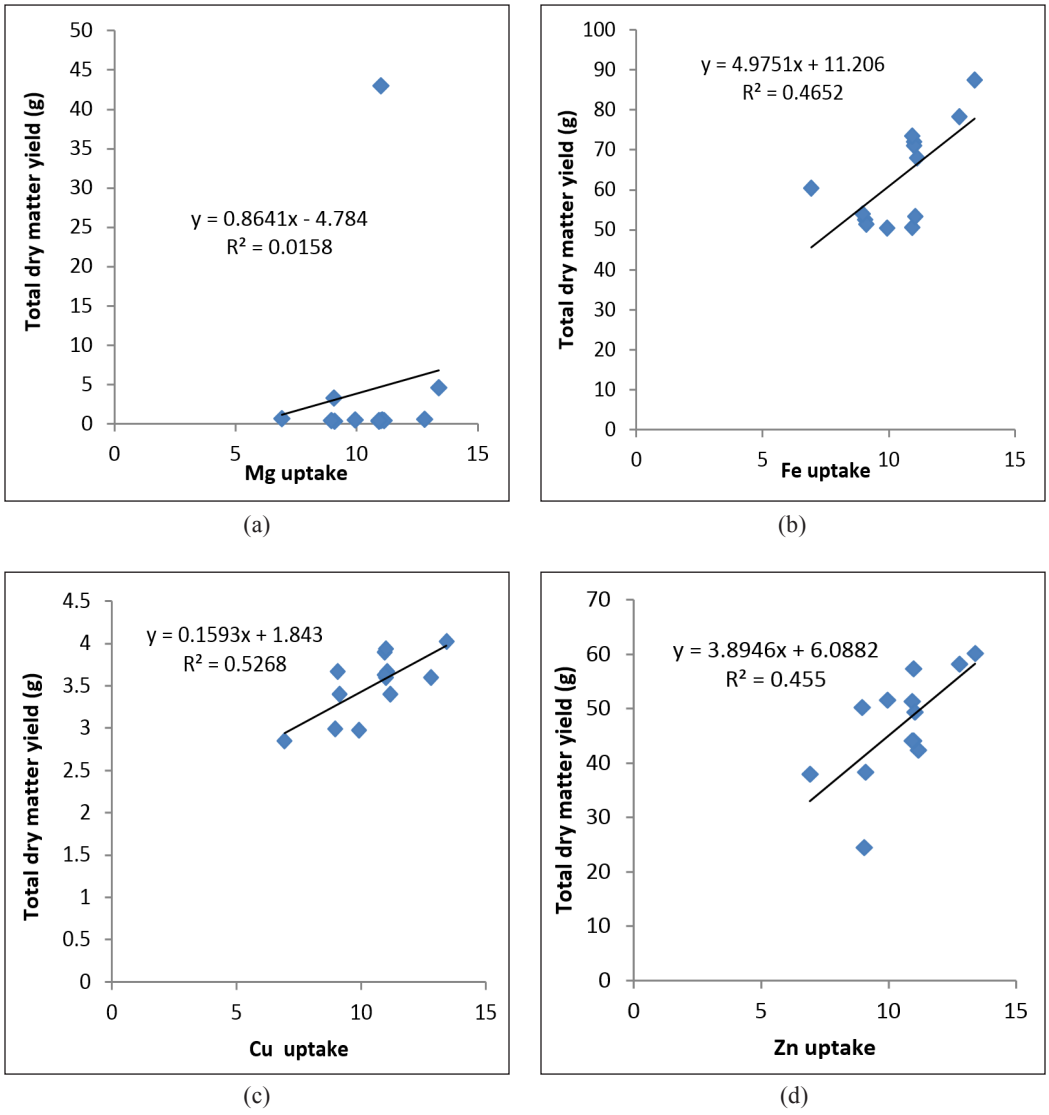

Figure S2. Correlation between total dry matter yield and nutrient uptake of (a) Mg; (b) Fe; (c) Cu; and (d) Zn

Figures 3 and 4 showed correlation between on total dry matter yield and available growth media nutrients There is a positive correction between the total biomass yield and the available growth media nutrients.

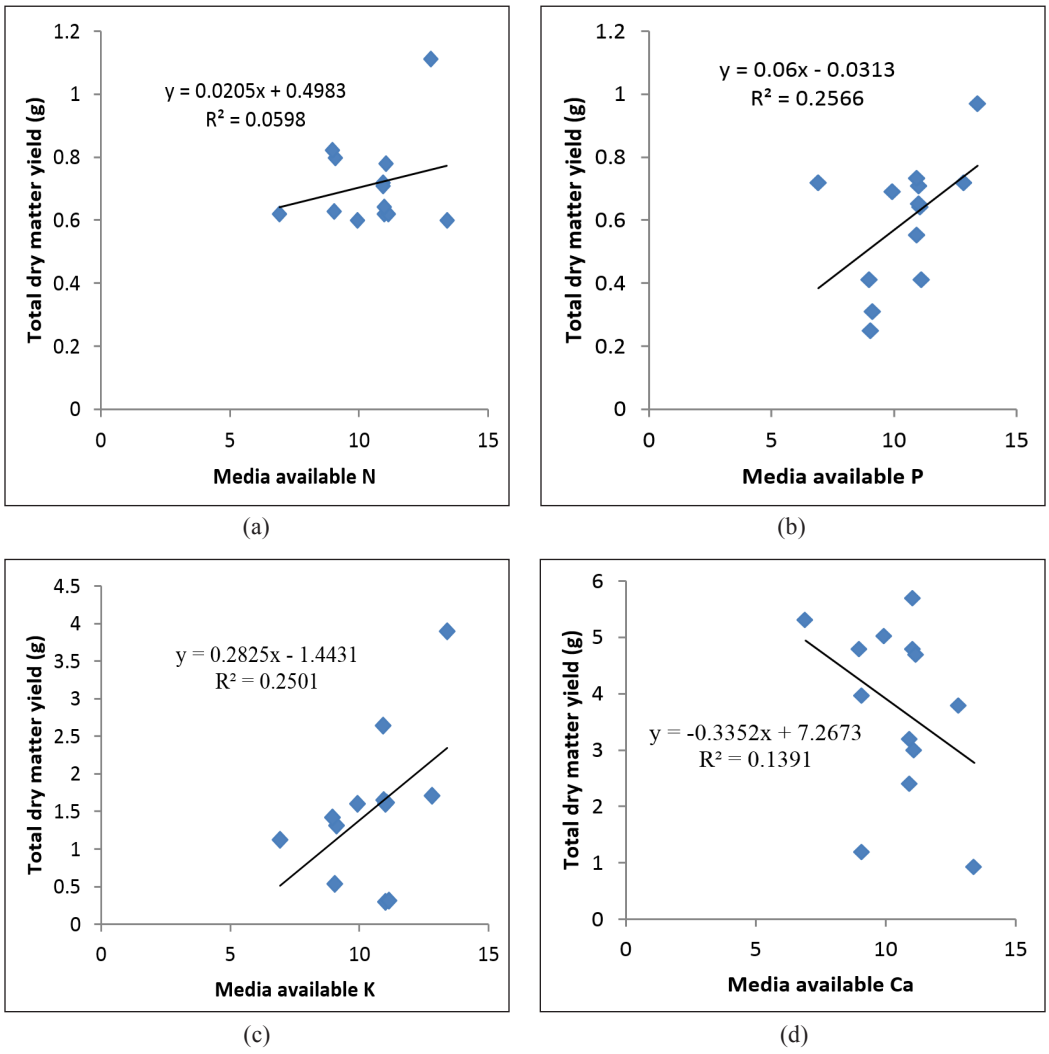

Figure S3. Correlation between total dry matter yield and available soil nutrient of (a) N: (b) P; (c) K; and (d) Ca

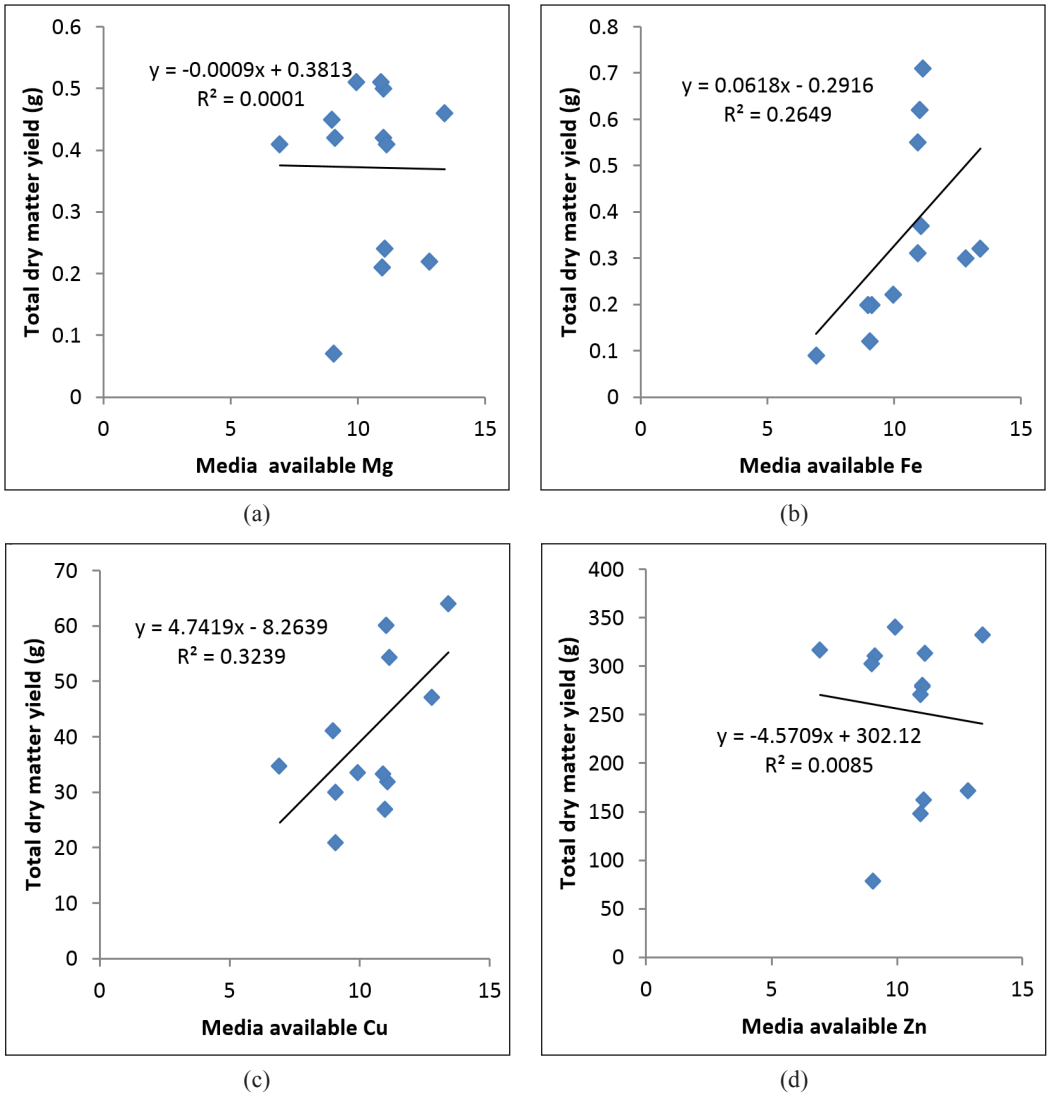

Figure S4. Correlation between total dry matter yield and available media nutrient content of (a) Mg; (b) Fe; (c) Cu; and Zn

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Effect Brassinolide Aplication on Growth and Physiological Changes in Two Cultivars of Fig (*Ficus carica* L.)

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ABSTRACT

Brassinolide (BL) is a plant hormone showing wide occurrence in the plant kingdom with unique biological effects on growth and physiological traits. The fig varieties, Improved Brown Turkey (IBT) and Masui Dauphine (MD), are commonly found in Indonesia and Malaysia. There is limited information on exogenous brassinolide application on these varieties. Thus, the aim of this study is to investigate the effect of different concentration of exogenous application of BL on growth and physiological changes of fig. Fig planting materials were propagated using stem cutting and then transferred into media containing 3:2:1 mixed soil (top soil: organic matters: sand). Two fig cultivars treated with BL (control, 50, 100 and 200 ml.L^{-1}) were arranged as Split Plot Randomized Complete Block Design (SRCBD) with four replications. Plant growth (Plant Height [PH], Total Leaf Area [TLA], Total Dry Biomass [TDB], Specific Leaf Area [SLA], Shoot to Root Ratio [S/R] and Net Assimilation Rate [NAR]) and physiological changes (Photosynthesis Rate [A], Stomatal Conductance [g_s], Transpiration Rate [E] and Chlorophyll Content [CC] were investigated every three weeks and at monthly intervals, respectively. Increasing BL concentration (50, 100, and 200 ml.L^{-1}) caused some differences in growth and physiological changes of fig, but the differences were not consistent and most of the changes happened only in first or second month. Cultivar IBT showed higher growth and physiological changes than cultivar MD after receiving brassinolide treatment. There was significant effect of interaction between brassinolide and variety on growth and physiological changes of fig

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except in the parameters of plant height and total dry biomass.

Keywords: Brassinolide, fig, growth, physiological changes

INTRODUCTION

Brassinolide (BL) is one of the brassinosteroids, which are the steroidal plant hormones that show a wide occurrence in the plant kingdom and have unique biological effects on growth and development (Clouse & Sasse, 1998; Khripach et al., 2000). They are a group of naturally occurring polyhydroxy steroids initially isolated from *Brassica napus* pollen in 1979. Research on brassinosteroids has revealed that they elicit a wide spectrum of morphological and physiological responses in plants that include stem elongation and cell division (Grove et al., 1979), leaf bending and epinasty (Sandalio et al., 2016). Besides their role in promoting plant growth activities, they also have physiological effects on the growth and development of plants (Khripach, et al., 2000; Vardhini, 2012).

Much has been written about BL. Clouse (2011), for example, pointed out that:

Among plant hormones, BL are structurally the most similar to animal steroid hormones, which have well-known functions in regulating embryonic and postembryonic development and adult homeostasis. Like their animal counterparts, BL regulate the expression of numerous genes, impact the activity of complex metabolic pathways, contribute to the regulation of cell division and differentiation, and help control overall developmental programs leading to morphogenesis. They are also involved in regulating processes more specific to plant growth including flowering and cell expansion in the presence of a potentially growth-limiting cell wall. (p. 1).

Fig (*Ficus carica* L.) belongs to the Moraceae family. It is a bush or small tree, moderate in size, deciduous with broad, ovate, three- to five-lobed leaves, contains copious milky latex and introduced to Indonesia and Malaysia from Middle East and Western Asia. There are over 700 named varieties of fig trees, but many of them are not grown in home garden (Carroll, 2015). Because fig seeds are non-viable, trees must be propagated via cuttings or grafts. Though the propagation of *F. carica* by vegetative cuttings insures uniformity, relatively low multiplication rates are achieved because these materials can be obtained only from upright branches, which results in poor rooting (Kumar et al., 1998); hence, brassinolide application was attempted by evaluating plant growth and physiological changes in *Ficus carica*.

In Malaysia and Indonesia, there are at least 21 known varieties of the fig tree and most of them are from Improved Brown Turkey (IBT) and Masui Dauphine (MD) varieties (Ahmad, 2012). There is limited information on exogenous brassinolide application on these varieties. Thus, the aim of this study was to investigate the effect of different concentrations of exogenous application of BL on growth

and physiological changes of fig var. IBT and MD.

MATERIALS AND METHODS

Fig-planting materials were propagated using cuttings taken from mature two- to three-year-old figs and transferred into media containing 3:2:1 mixed soil (top soil: organic matters: sand). Two different fig (IBT and MD) varieties were subjected to four levels (0, 50, 100 and 200 $ml.L^{-1}$) of BL concentration. One-month-old fig tree seedlings were sprayed monthly with a solution of brassinollide (tetrahydroxy-methyl-B-homo-oxa-cholestan-lactone + Multi Purpose Cultivation [MPC] + water) according to the treatments. Fig varieties were considered as the main treatment and BL concentrations (B) as sub-treatments. The experiment was arranged in Split Plot Randomised Complete Block Design (SRCBD) with four replications. There were four plants as destructive samples observed monthly for each replication. The experiment was conducted in an open field at Ladang 15, Faculty of Agriculture, Universiti Putra Malaysia situated at 2° 58" N and 101° 44' 04" E in Serdang, Selangor, Malaysia. Data were recorded weekly and monthly.

Growth Measurements

Determination of Plant Height (PH). Plant height was measured using a ruler as the distance between the soil and the shoot apex.

Determination of Total Leaf Area per Seedling (TLA). Total leaf area per plant was measured using a leaf area meter (Model LI –3100A Lincoln Inc., Nebraska, USA). The leaves were passed between an array of light sensors and the total area was estimated from the occlusion of light by the leaf. The leaves were placed in polythene bags and kept in the refrigerator (6°C in darkness) for no longer than 12 hours before measuring the leaf areas (Jaafar, 1995). Detached leaves were then passed through the instrument, which was calibrated using a standard calibration plate with an area of about 100 cm^2. The leaves were arranged in the field within view. Overlapping of adjacent leaves was avoided. The mean value of three plant samples were used to represent each experimental unit.

Determination of Total Dry Biomass (TDB). Total dry matter accumulation per plant was taken by calculating the dry weight of the roots, stem and leaves. Prior to drying, the plants were separated into leaves, stem and roots. The plant parts were placed in paper bags and oven-dried at 45 ^0C until constant weight (i.e. three days) was reached. Plant total dry weight was taken using a sensitive electronic weighing scale (Model CDS 125, Mitutoyo Inc., Japan).

Determination of Specific Leaf Area (SLA). The SLA measures the leafiness of the plant on dry weight basis (Henson, 1995).

Determination of Shoot to Root Ratio (S/R). S/R of the seedling was determined to know the partitioning of dry matter of the

plant. The S/R was determined using the Hunt equation (Hunt, 1990).

Determination of Net Assimilation Rate (NAR). Values of NAR were measured using the Beadle formula (Beadle, 1998).

Physiological Measurements

Determination of Photosynthesis Rate (A), Stomatal Conductance (g_s) and Transpiration Rate (E). Photosynthetic rate, stomatal conductance and transpiration rate of fully expanded leaves were measured using a portable photosynthesis system (LICOR–6400, Inc., USA). Prior to use, the instrument was warmed and calibrated for 30 min on ZERO IRGA mode. The measurements of gas exchange were carried out between 0900 and 1100.

Determination of Chlorophyll Content (CC). Total chlorophyll content was measured using the method of Idso et al. (1996) on fresh weight basis.

Statistical Analysis

All the data obtained were analyzed using Statistic Analysis System (SAS) version 9.4. Significant difference in mean values were determined and analyzed using two-way ANOVA and the mean differences were compared using the Least Significand Different Test (LSD) at 5% and 1% level of significance.

RESULTS

Effect Brassinolide on Growth of Fig

The growth of the fig plants was affected by the brassinolide levels. Treatment of the fig plants with different concentrations of brassinolide (50, 100 and 200 $ml.L^{-1}$) caused an increase in plant height and total dry biomass compared to control samples. Total leaf area, specific leaf area and shoot-to-root ratio increased with increasing concentrations of brassinolide up to 100 $ml.L^{-1}$, followed by a decline whereas net assimilation rate fluctuated over a period of study. At the first Month After Treatment (MAT), increasing brassinolide concentration (50 and 100 $ml.L^{-1}$) caused an increase in the net assimilation rate when compared to control but there was a decrease when brassinolide concentration was 200 $ml.L^{-1}$. At the second MAT, by increasing the brassinolide concentration (50, 100 and 200 $ml.L^{-1}$), the net assimilation rate had decreased.

Application of brassinolide had some effect on plant height, total leaf area, total dry biomass, specific leaf area and net assimilation rate (Table 1) but it was not significant on the shoot-to-root ratio. Among the varieties, IBT showed higher growth than MD at every five-weekly observation. There was a significant interaction between the brassinolide and the cultivar for total leaf area, specific leaf area, shoot-to-root ratio and net assimilation rate parameters. Additionally, only shoot-to-root ratio parameter showed a significant effect of interaction between the brassinolide and cultivar at 1% level of significance.

Table 1

Effect of different concentrations of brassinolide on growth of two cultivars of fig

Treatments	Plant Height (cm)					Total Leaf Area (cm²)				Total Dry Biomass (g)			
	Week After Treatment					Month After Treatment				Month After Treatment			
	3	6	9	12	15	1	2	3	4	1	2	3	4
Control	16.44	26.34	32.43	36.83	*41.85b	*295.81ab	298.14	468.71	284.61	5.46	7.85	33.44	35.46
50ml/L	15.70	23.16	33.87	38.06	*44.39ab	*89.94b	294.89	416.22	468.67	4.07	7.86	31.81	49.35
100ml/L	17.26	24.82	33.76	39.39	*38.17b	*176.33ab	320.77	367.95	314.47	5.38	11.72	30.33	35.98
200ml/L	16.87	25.39	34.16	44.93	*51.21a	*385.92a	479.70	468.51	430.61	7.12	9.95	45.17	53.86
IBT	*19.41a	**27.74a	*36.50a	40.24	43.52	312.82	397.04	*467.65a	*303.04b	*6.92a	*10.76a	*37.32a	43.60
MD	*13.72b	**22.12b	*30.62b	39.36	44.28	161.19	299.71	*393.04b	*446.14a	*4.09b	*7.92b	*33.05b	43.72
IBT + 50ml/L	17.76	24.88	36.24	37.89	45.57	89.89	421.18	*419.61ab	429.83	3.92	10.33	37.86	56.11
IBT + 100ml/L	20.71	27.84	36.56	37.40	36.14	322.27	375.60	*222.36b	179.05	7.74	13.99	24.25	32.66
IBT + 200ml/L	17.89	26.70	36.56	44.63	48.42	359.42	450.05	*704.15a	352.39	9.14	9.07	50.12	42.15
MD + 50ml/L	13.63	21.45	31.51	38.23	43.20	90.00	168.61	*412.82a	507.51	4.23	5.38	25.76	42.59
MD + 100ml/L	13.81	21.81	30.96	41.37	40.20	30.40	265.95	*513.55a	449.89	3.01	9.45	36.41	39.31
MD + 200ml/L	15.85	24.09	31.76	45.23	54.00	412.43	509.36	*232.88a	508.83	5.10	10.83	40.22	65.57
LSD V	1.31	1.93	4.25					73.57	90.54	2.17	2.69	4.05	
LSD B					9.28	279.29							
LSD V*B								479.04*504.01					

Treatments	Specific Leaf Area (cm²/g)				Shoot-to-Root Ratio				Net Assimilation Rate (g/cm²/month)			
	Month After Treatment				Month After Treatment				Month After Treatment			
	1	2	3	4	1	2	3	4	1	2	3	4
Control	11.96	8.06	8.43	4.40	*2.95b	9.65	*3.67b	*3.55ab	*0.27b	*0.54a	*0.48b	1.03
50ml/L	7.45	9.64	7.07	5.19	*4.13ab	2.89	*3.10b	*3.01b	*0.41a	*0.24b	*0.58b	0.69
100ml/L	7.97	7.37	6.22	4.76	*2.44b	3.78	*2.62b	*3.99a	*0.42a	*0.39ab	*0.58b	1.09
200ml/L	13.73	9.99	7.83	5.16	*5.83a	7.54	*4.41a	*4.01a	*0.13b	*0.49a	*0.91a	0.84
IBT	11.04	8.36	*6.84b	*4.33b	3.54	5.06	3.49	3.42	0.30	0.55	*0.56b	1.00
MD	9.51	9.17	*7.94a	*5.43a	4.13	6.86	3.41	3.86	0.31	0.27	*0.71a	0.82
IBT + 50ml/L	*7.10a	11.45	5.99	4.42	4.25	2.50	3.29	**3.08bc	*0.55a	*0.15b	0.60	0.81
IBT + 100ml/L	*12.69a	8.42	5.58	3.66	2.76	3.23	2.64	**2.57c	*0.16a	*0.38ab	0.58	1.09
IBT + 200ml/L	*8.96a	9.10	8.18	5.48	4.76	11.10	3.51	**4.10a	*0.17a	*0.73ab	0.50	0.88
MD + 50ml/L	*7.79b	7.82	8.15	5.95	4.00	3.28	2.90	**2.94b	*0.27b	*0.34a	0.57	0.56
MD + 100ml/L	*3.25b	6.33	6.86	5.86	2.13	4.33	2.61	**5.42a	*0.67a	*0.40a	0.57	1.09
MD + 200ml/L	*18.49a	10.88	7.48	4.84	6.89	3.97	5.31	**3.92ab	*0.09b	*0.24a	1.32	0.79
LSD V			0.88	0.62							0.14	
LSD B					2.74		0.85	0.89	0.12	0.11	0.29	
LSD V*B	13.25*9.07							0.91*1.70	0.55*0.41	0.76*0.41		

Means followed by the different small letters are significant at *=p<0.05, **=p<1%.

Effect of Brassinolide on Physiological Changes of Fig

Table 2 shows that the physiological changes of fig were affected by the brassinolide levels and the cultivars. Interaction between brassinolide concentrations and fig variety was significant only at 5%. Similar to morphological parameters, physiological traits such as photosynthesis, transpiration rate, and chlorophyll have shown some differences with brassinolide application, but the differences were not consistent and most of the changes happened only in first or second month. Both the brassinolide and the cultivar treatments were effective on the physiological changes of fig except on stomatal conductance.

Varietal performance of brassinolide application was analyzed at specific period of the study and the result is presented in Figures 1 and 2. Increasing concentration of brassinolide (50, 100 and 200 ml.L^{-1}) had decreased the rate of photosynthesis, transpiration and chlorophyll content in IBT than MD.

Correlation analysis was carried out to establish the relationship between the parameters. Figure 3 shows a significant positive inter-correlation among parameters such as chlorophyll content, specific leaf area, transpiration rate and stomatal conductance. Increase in chlorophyll content, transpiration rate,

total dry biomass, photosynthetic rate, and total dry biomass was associated with an increase in specific leaf area, transpiration rate, stomatal conductance, net assimilation rate and total leaf area with an r value of 14.95%, 27.75%, 3.97%, 62.08%, 36.93%, 25.27% and 21.13%, respectively.

Significant negative correlation was noted between total dry biomass with specific leaf area; total dry biomass with transpiration rate; transpiration rate with net assimilation rate; chlorophyll content with net assimilation rate; and specific leaf area with net assimilation rate. Increase in total dry biomass, transpiration rate, chlorophyll content and specific leaf area was associated with a decrease in specific leaf area, transpiration rate and net assimilation rate with an r value of 24.18%, 13.31%, 12.75%, 14.45%, and 49.25%, respectively.

DISCUSSION

We studied the effect of exogenous brassinolide application on some growth and physiological traits on two cultivars of fig. The main functions of brassinollide are to promote the plant growth especially for cell elongation and division (Mayumi & Shibaoka, 1995) and has the ability to stimulate other physiological processes (Prusakova et al., 1999).Wang et al. (1993) had found that brassinollide appeared to cause elongation by affecting wall extensibility and increasing wall relaxation properties.

As levels of brassinolide increased (50, 100 and 200 ml.L^{-1}), plant height, leaf area, total dry biomass and net assimilation rate parameters also linearly improved at 28%, 25%, 6% and 66%, respectively, higher than recorded for the control treatment. Similar results were reported by other researchers for other plants i.e. Hu et al. (2013) for

Table 2

Effect of different concentrations of brassinolide on physiological changes of two cultivars of fig

Treatments	Photosynthesis Rate ($\mu mol.m^{-2}s^{-1}$)				Stomatal Conductance ($mmol\ m^{-2}\ s^{-1}$)			
	Month After Treatment				Month After Treatment			
	1	2	3	4	1	2	3	4
Control	*13.31a	11.99	16.96	20.29	0.67	0.22	0.56	0.34
50ml/L	*10.66b	10.94	17.35	23.45	0.63	0.26	0.41	0.36
100ml/L	*9.69b	10.81	16.19	23.40	0.40	0.23	0.31	0.22
200ml/L	*10.65b	11.07	17.63	23.16	0.42	0.20	0.53	0.32
IBT	11.30	*11.82a	17.28	22.00	0.52	*0.26a	*0.54a	0.32
MD	10.86	*10.59b	16.79	23.15	0.54	*0.20b	*0.37b	0.30
IBT + 50ml/L	10.57	*12.16ab	18.19	23.06	*0.74a	0.31	0.41	0.52
IBT + 100ml/L	11.64	*13.19a	13.18	23.54	*0.50a	0.28	0.29	0.24
IBT + 200ml/L	9.55	*7.94b	20.36	22.22	*0.32a	0.18	0.71	0.33
MD + 50ml/L	10.75	*9.73a	16.51	23.84	*0.52ab	0.21	0.40	0.20
MD + 100ml/L	7.73	*8.44a	19.19	23.27	*0.30b	0.18	0.33	0.19
MD + 200ml/L	11.75	*14.20a	14.90	24.10	*0.51ab	0.23	0.35	0.31
LSD V		1.22				0.04	0.29	
LSD B	2.45							
LSD V*B		4.72*8.22			0.63*0.46			

Treatments	Transpiration Rate ($mol\ m^{-2}\ s^{-1}$)				Chlorophyll Content (mg/g fresh weight)			
	Month After Treatment				Month After Treatment			
	1	2	3	4	1	2	3	4
Control	*5.00a	3.33	*3.59a	3.22	18.79	4.73	*14.22b	4.20
50ml/L	*4.51ab	3.54	*3.07b	2.80	18.38	4.92	*14.27b	4.39
100ml/L	*3.50b	3.22	*2.82b	2.54	18.90	4.76	*14.24b	4.28
200ml/L	*3.58b	2.86	*3.69a	3.24	18.90	5.09	*14.35a	4.89
IBT	3.92	*3.47a	*3.57a	2.99	**19.07a	*4.96a	14.26	4.57
MD	4.38	*3.01b	*3.01b	2.90	**18.41b	*4.80b	14.28	4.31
IBT + 50ml/L	4.47	*3.85a	3.48	3.25	*18.91a	5.03	14.24	4.44
IBT + 100ml/L	3.76	*3.76a	3.04	2.69	*19.06a	4.65	14.23	4.36
IBT + 200ml/L	3.05	*2.42b	3.78	3.25	*19.05a	5.06	14.38	5.20
MD + 50ml/L	4.55	*3.22a	2.66	2.35	*17.85b	4.81	14.29	4.35
MD + 100ml/L	3.24	*2.69a	2.59	2.39	*18.75a	4.87	14.25	4.20
MD + 200ml/L	4.11	*3.31a	3.60	3.23	*18.75a	5.12	14.31	4.57
LSD V		0.45	0.53		0.41	0.13		
LSD B	1.03		0.47				0.07	
LSD V*B		1.33*2.16			0.80*0.82			

Means followed by the different small letters are significant at *=p<0.05, **=p<1%.

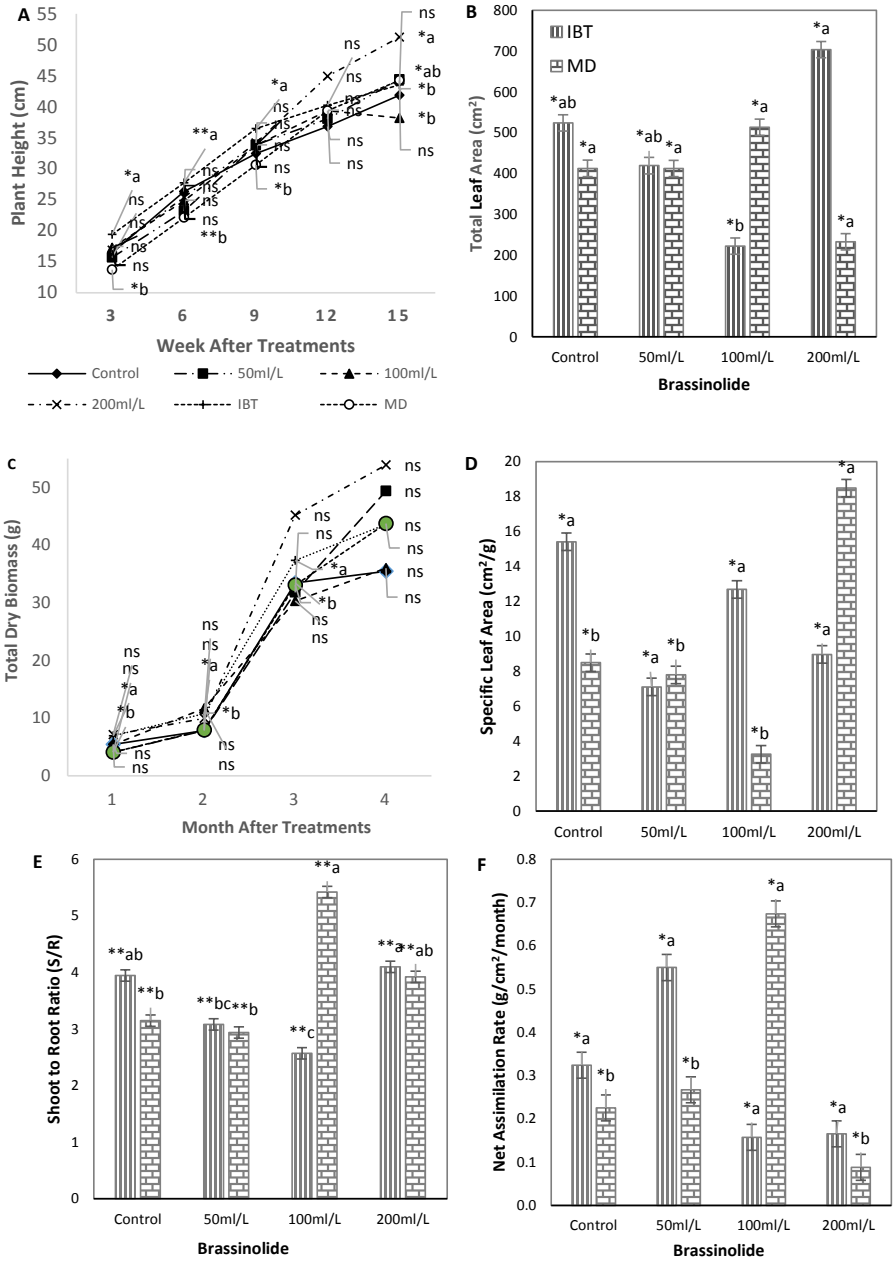

Figure 1. Significant growth of fig according to parameters: (A) Plant height as main effect of brassinolides on the cultivars; (B) TLA at third MAT as interaction between cultivars and brassinolide; (C) TDB as main effect of brassinolides and cultivars; (D) SLA at first MAT as interaction between cultivars and brassinolides.E) S/R at fourth MAT as interaction between cultivars and brassinolides; NAR as interaction between cultivars and brassinolides at: (F) First MAT. Bars and curves represent means followed by the different small letters are significant at *=p<0.05, **=p<1%, and ns=not significant

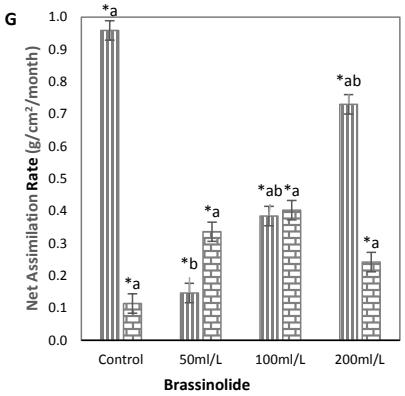

Figure 1. Significant growth of fig according to parameters: (G) Second MAT. Bars and curves represent means followed by the different small letters are significant at *=p<0.05, **=p<1%, and ns=not significant

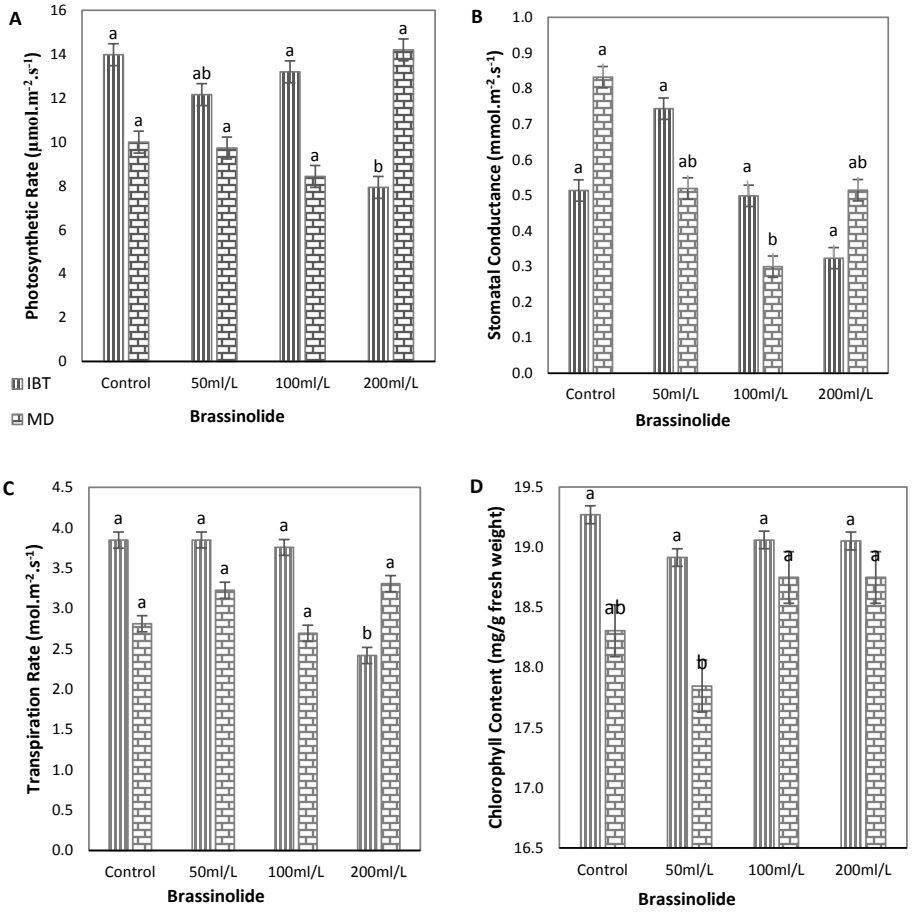

Figure 2. Significant physiological changes of fig according to parameters: (A) A at second MAT; (B) g_s at first MAT; (C) E at second MAT; and (D) CC at first MAT. Bars represent means followed by the different small letters significant at p<0.05

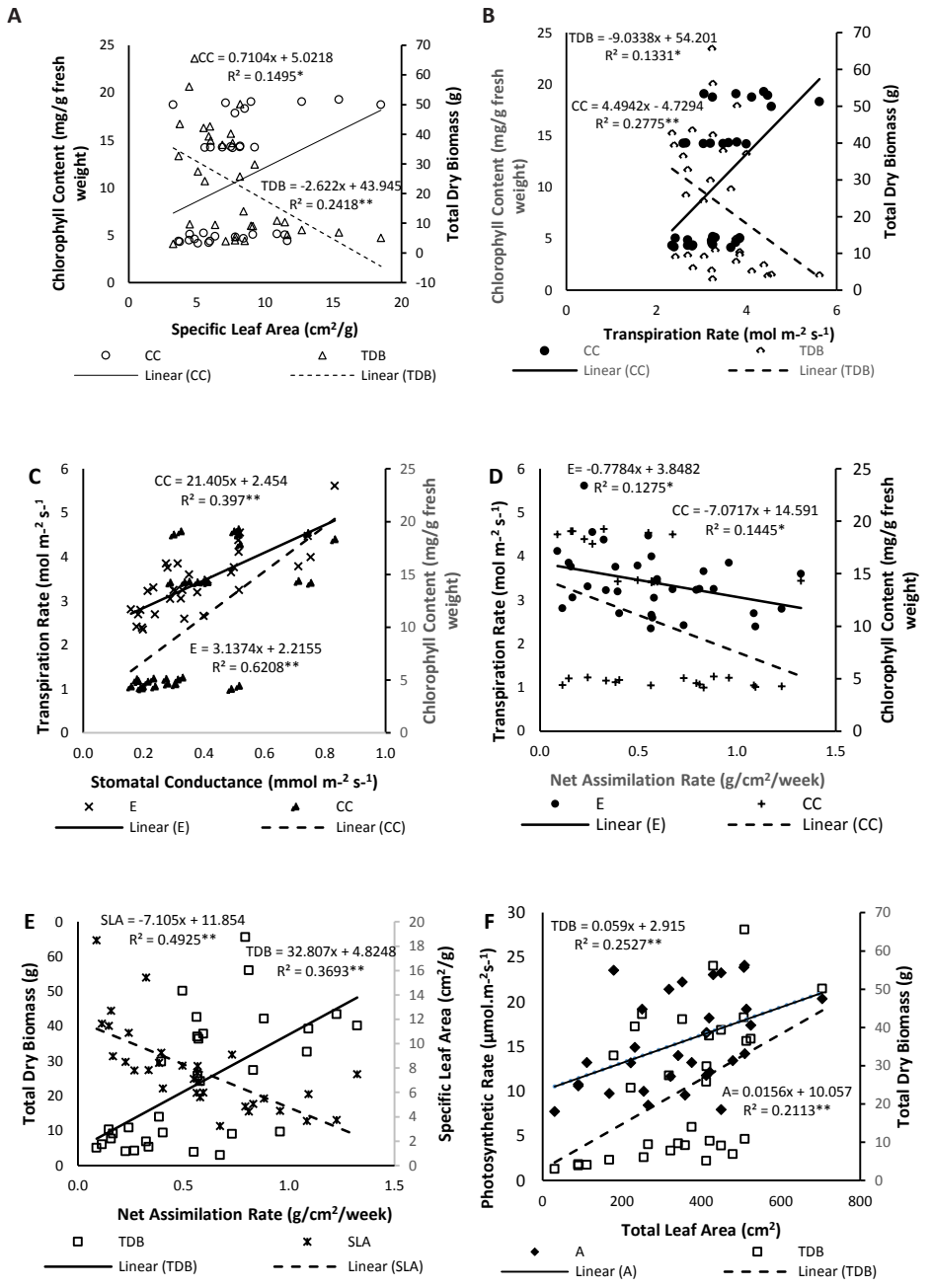

Figure 3. Correlation coefficient between CC and TDB with (A) SLA; (B) E; E and CC with (C) g_s; (D) NAR; TDB and SLA with (E) NAR; (F) TLA. *= p≤ 0.05, **=p ≤0.01, n=128

Leymus chinensis; Bera et al. (2014) for sunflower; and Anjum et al. (2011) for maize. The growth stimulation was more pronounced on above ground biomass than below ground biomass, showing a high shoot-to-root ratio (Zaharah et al., 2006). The increase in growth in this study might have been due to increased carboxylation rate after using the BL treatment, which enhanced carbon assimilation, channeling it to stimulate increase in plant height, leaf area and total biomass (Henson, 1992).

Specific leaf area (SLA) is one growth parameter that characterized the thickness of the leaves. Usually plant with high SLA had the thinnest leaves. Specific leaf area was found to be lower than the control ($p \leq 0.05$) under brassinolide concentrations of 50 and 100 $ml.L^{-1}$. The result implies that plants have thicker leaves. The thicker leaf might have been due to increase in the mesophyll layer after receiving brassinolide (Haniff, 2006). The increase in leaf thickness could also have been due to higher leaf weight ratio in fourth MAT compared with first to third MAT. The leaf area was maintained at lowest SLA. That indicated that leaves of fig were thickest at brassinolide 100 $ml.L^{-1}$. This indicated that increase in SLA was due to increase in leaf weight compared with increase in leaf area (Hayat et al., 2012; Lambers & Poorter, 1992).

The net assimilation rate (NAR) of plants are growth characteristics that best describe plant growth performance under specified conditions (Gardner et al., 1994). It is evident that plants under elevated BL have high NAR. Increase in plant growth grown under different planting geometries and depths in SRI has also been reported by Rajput et al. (2017), who reported that increase in total biomass by 30% in rice had increased NAR by 4% compared with the control. The reduction in NAR was due to the ontogenical development of fig.

Brassinolide (BL) had profound impact on leaf photosynthesis and plant performance. Brassinolide (BL) improved leaf carbon assimilation rate, which is the light harvesting machine of plant photosynthesis. Brassinolide (BL) treatment also enhanced photosynthetic performance of cotton seedlings under NaCl stress (Chen et al., 2007; Shu et al., 2011; Xiao et al., 2007). For cucumber seedlings, BL treatment has also been found to promote the occurrence of new roots, the formation of lateral roots and nutrient uptake (Bao et al., 2004).

Brassinolide (BL) treatment enhanced photosynthesis (17.06%) and chlorophyll content (18.36%). In contrast, BL-treatment decreased stomatal conductance (11.94.50%) and transpiration rate (17.83%). The BL-induced increase in photosynthesis could have been due to improvements in leaf-water balance as indicated by increased water potential (Sairam, 1994) and improved chlorophyll content and higher leaf area in BL-treated plants (Iwahari et al., 1990).

Stomata are the windows that admit water and CO_2 in and out of the plant. Chlorophyll content and transpiration rate were found to have declined. This could be attributed to the enhanced growth of

seedlings under elevated BL treatment that diluted the nitrogen content in the plant tissue (Ibrahim et al., 2011). Figures 3A and C showed a significant positive inter-relation among chlorophyll content, transpiration rate and stomatal conductance, indicating that a decrease in chlorophyll content would associated with same degree of reduction in transpiration rate and stomatal conductance.

CONCLUSION

Brassinolide application had brought notable changes in growth and physiology among fig varieties. Though increasing BL concentration (50, 100 and 200 $ml.L^{-1}$) caused some differences in growth and physiological changes of fig, but the differences were not consistent and most of the changes happened only in first or second month. Cultivar IBT showed higher growth and physiological changes than cultivar MD after receiving brassinolide treatment. There was significant effect of interaction between brassinolide and variety on growth and physiological changes of fig except for plant height and total dry biomass. In the future, the experiment would be repeated in a greenhouse under controlled environment to verify the effect of brassinolide on fig varieties.

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Robust Assessment of Body Weight and Linear Body Measurements of Nigerian Normal Feather Chickens using Bayesian Inference

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ABSTRACT

Previous studies on the relationships between body weight and morphometric indices in chickens have been mainly on classical analysis with assumptions that, data have normal distribution and constant variances. A more reliable assessment of body weight and morphometric indices requires a Bayesian multiple linear regression with assumptions of unequal variances. Body weight and nine morpho-structural traits of 234 Nigerian indigenous normal-feather chickens were measured using weighing scale and measuring tape at sixteen weeks. Two different regression models (weighted and unweighted) were fitted in Winbugs software to obtain Bayesian inference for each sex. Predicted relationships between body weight and shank length, thigh length, keel length, body length, wing length and breast girth were positive and ranged from 0.272 ± 4.972 to 101.5 ± 24.56. Shank diameter, tail length and wing span had negative relationships with body weight and estimates ranged from –15.94 ± 12.31 to –4.608 ± 59.86. Goodness of fit of models was assessed using Bayesian p–value, Deviance information criterion (DIC) and graph of residuals against predicted values under each model. The Bayesian p–value (0.502) for unweighted model for male chicken was closed to 0.5 compared to its weighted counterpart (0.573). This implied that weighted model fitted relationship between body weight and morpho-structural traits in Nigerian normal-feather male chicken compared to unweighted model. The

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differences in DIC and Bayesian p–values and residuals' plot against predicted values of weighted and unweighted regression models were sufficient for us to believe that weighted models fitted body weight and morpho-structural traits data better than unweighted models.

Keywords: Bayesian, body dimension, indigenous, model

INTRODUCTION

Chicken is the most common among poultry growers in Nigeria. Many farms have started to raise local chickens at commercial level due to growing interest as providers of meat and eggs. Nigerian indigenous chickens (NIC) contain a highly conserved genetic reservoir, with high level of heterozygosity, which may serve as biological animals, and offer useful information on the suitability of animals for selection (Ajayi et al., 2012). Normal-feather chicken is among NIC that provides meat, eggs and even manure at subsistence level to rural people.

Relationship exists between body weight and linear body measurements (Ige, 2013). Linear body measurements serve as good indicator of body weight and market value of chickens apart from body weight (Ukwu et al., 2014). Hence, the increasing need to estimate the weight of chickens in order to study their growth pattern as resulted in development of different regression equations. These equations are designed to predict the live weight of animals from linear body measurements (Peters et al., 2007). Assessment of body weight and linear body measurements of chickens have been reported by several authors in literature (Alabi et al., 2012; Gueye et al., 1998; Ibe, 1989; Ibe & Ezekwe, 1994; Ige et al., 2007; Ige, 2013; Momoh & Kershima, 2008; Ukwu et al., 2014). This assessment was based on frequentist (classical) predictions where solution of a model consisted in a single value for each parameter while as Bayesian predictions are based on estimation of distribution rather than single value. Despite this shortcoming in classical prediction, no available information on use of Bayesian inferences to study relationships of body weight and linear body measurements exist. In classical predictions, formula for estimating standard error is more complicated, including the Gaussian multipliers for regressions that contain more than one explanatory variable. This complex formula can be ignored when using Bayesian methods (McCarthy, 2007).

Therefore, in this study, unweighted and weighted Bayesian regression analyses were used to predict body weight from linear body measurements in Nigerian indigenous normal feather chicken. In unweighted model, we assumed that dependent variable is normally distributed, and its variance is constant over all values of the independent variables while in weighted model, we assumed unequal variances over all values of the independent variables. This is done so that we can have robust assessment of relationship between body weight and linear body measurements in normal-feather chicken.

MATERIALS AND METHODS

Location of Study, Experimental Birds and their Management

The research was carried out in Alabata, Abeokuta, Ogun State, located on latitude 7˚10 N in Odeda Local Government Area, Ogun State, in South–Western Nigeria. The ambient temperature during the period ranged from 26.9^{o}C in June to 27.1^{o}C in December with average relative humidity of 80%, while the vegetative site represents an inter–phase between the tropical rainforest and the derived savannah. Fertile eggs of Nigerian indigenous chicken were collected from the Poultry Breeding Unit of Federal University of Agriculture, Abeokuta and hatched at the hatchery of the Unit. Two hundred and thirty-four chicks (95 males and 139 females) were collected from the hatchery at day–old and raised up to sixteen weeks of age. The chicks managed under intensive system and fed commercial feed purchased from market and water supplied *ad libitum*. The diet fed from day-old to 8th weeks had 2800 kcal of ME/kg of diet with 22% CP. Also, the diet fed from 9th to 16th weeks had 3000 kcal of ME/kg of diet with 20% CP.

Data Collection

Body weight and nine linear body measurements of 234 Nigerian Indigenous normal feather chickens were individually measured using a 5kg weighing instrument with sensitivity of 0.01 g and a measuring tape. Reference points for body measurement were according to standard descriptor (Sørensen, 2010). The parts measured were body length (BOL), measured as the distance between the tip of the beak and the longest toe without the nail; wing length (WIL), taken as the distance between the tip of the phalanges and the coracoids-humerus joint; wing span (WIS), measured as the distance between the left wing tip to the right wing tip across the back of the chicken; shank length (SHL), taken as the distance from the hock joint to the tarsometatarsus; thigh length (THL) measured as the distance between the hock joint and the pelvic joint; breast girth (BOG), measured as the circumference of the breast around the deepest region of the breast and keel length (KEL), taken as the distance between the anterior and posterior ends of the keel, shank diameter (SHD) measured as the circumference of the shank at the middle region and tail length (TAL) measured as the length from the tip of a central rectrix to the point where it emerged from the skin.

Statistical Analysis

Bayesian analysis of variance was carried out to estimate means and standard errors of body weight and linear body measurements for both sexes. The 95% Highest Density Interval and Bayes factor values were used as critical to determine significant difference between the sexes using R software. In order to carry out robust Bayesian regression analysis, two different regression models (weighted and unweighted) were fitted in Winbugs software to obtain Bayesian inference for each sex. The unweighted model assumes body weight must be normally distributed, and the variance of

the body weight must be constant over all values of the linear body measurements while weighted model assumes unequal variances over all values of the linear body measurements. The general model is given below:

$$Y_{ij} = b0 + b1*SHL + b2*SHD + b3*THL + b4*KEL + b5*BOL + b6*TAL + b7*WIL + b8 * WIS + b9 * BOG + e_{ij}$$

Prior distributions for regression coefficients in the model above are listed below:

tau ~ dgamma (0.01, 0.01)
b0 ~ dnorm (0.0, 0.0001)
b1 ~ dnorm (0.0, 0.0001)
b2 ~ dnorm (0.0, 0.0001)
b3 ~ dnorm (0.0, 0.0001)
b4 ~ dnorm (0.0, 0.0001)
b5 ~ dnorm (0.0, 0.0001)
b6 ~ dnorm (0.0, 0.0001)
b7 ~ dnorm (0.0, 0.0001)
b8 ~ dnorm (0.0, 0.0001)
b9 ~ dnorm (0.0, 0.0001)

where tau is precision, which is inverse of variance of the model, b0 is intercept, b1 is shank length regression coefficient, b2 is shank diameter regression coefficient, b3 is thigh length regression coefficient, b4 is keel length regression coefficient, b5 is body length regression coefficient, b6 is tail length regression coefficient, b7 is wing length regression coefficient, b8 is wing span regression coefficient; b9 is breast girth regression coefficient for linear body measurements and Y_i is body weight. A posterior analysis was executed with 51,000 observations generated for the simulation, with a burn in of 5000 and a refresh of 100 in WinBUGS version 1.4.3 (Spiegelhalter et al., 2003) package. Posterior predictive check with a Bayesian p–value to assess the adequacy of the model for the dataset was included in the code. The code for setting up this model in WinBUGS is available on demand from the authors.

RESULTS AND DISCUSSION

Table 1 shows the means, standard errors and Bayes factor of body weight and linear body measurements of Nigerian indigenous normal-feather chicken at sixteen weeks. Based on Bayes factor values and 95% HDI (HDI tables not shown), male chicken had credibly better body weight and body measurements than female chickens. Kass and Raftery (1995) suggested that if Bayes factor value was less than 3 it was not worth mentioning, while Bayes factor value from 3 up to 20 are positive evidence for significant difference. Bayes factor value from 20 up to 150 is strong evidence, and more than 150 are very strong evidence for the significant difference. Hence, multiple linear regressions were carried out for female and male separately in order to avoid interference of sex effect as confounding factor in the analysis. Multiple linear regressions were used to test for the nature of relationship between body weight and linear body measurements. Table 2 showed unweighted multiple regressions relating body weight to linear body measurements

Table 1
Effect of sex on body weight and linear body measurements of Nigerian normal feather male chickens using Bayesian inference

Variable	Sex	Mean ± SE	N	Bayes Factor
Body weight	Female	1404.554± 16.359	138	4.371×10^{24}
	Male	1805.927 ± 16.434	95	
Shank length	Female	9.987 ± 0.057	138	3.251×10^{14}
	Male	11.040 ± 0.058	95	
Shank diameter	Female	4.907 ± 0.018	138	1.513×10^{16}
	Male	5.277 ± 0.018	95	
Thigh length	Female	15.409 ± 0.086	138	6.109×10^{15}
	Male	17.053 ± 0.087	95	
Keel length	Female	11.657 ± 0.052	138	8.224×10^{10}
	Male	12.493 ± 0.053	95	
Body length	Female	61.059 ± 0.302	138	1.415×10^{23}
	Male	68.292 ± 0.305	95	
Tail length	Female	18.403 ± 0.170	138	7.261×10^{3}
	Male	20.010 ± 0.171	95	
Wing length	Female	23.651 ± 0.114	138	8.248×10^{13}
	Male	25.679 ± 0.114	95	
Wing span	Female	81.449 ± 0.356	138	1.748×10^{14}
	Male	87.820 ± 0.355	95	
Breast girth	Female	29.7267 ± 0.130	138	3.925×10^{15}
	Male	32.162 ± 0.131	95	

SE = standard error, N = sample size

Table 2
Unweighted multiple regression relating body weight to linear body measurements of Nigerian normal feather male chickens

Parameters	Reg. coeff.	sd	MC error	95% credible interval
Intercept	–162.9	96.89	3.327	–349 to 24.19
Shank length	101.5	24.56	1.459	56.84 to 154.8
Shank diameter	–4.608	59.86	3.642	–120.4 to 116
Thigh length	10.74	17.21	1.019	–24.31 to 46.3
Keel length	60.87	27.82	1.726	2.888 to 116.2
Body length	5.619	4.38	0.2603	–3.004 to 14.42
Tail length	–6.126	6.999	0.3112	–19.73 to 7.788
Wing length	–15.94	12.31	0.76	–40.97 to 9.371
Wing span	0.272	4.972	0.3207	–9.805 to 10.19
Breast girth	1.861	11.89	0.7494	–22.13 to 24.93
bpvalue	0.5021	0.5	0.002502	0 to 1
tau	$3.30(10^{-5})$	$5.15(10^{-6})$	$9.83(10^{-8})$	2.37 to $4.38(10^{-5})$

Reg. coeff = Regression coefficient, sd = standard deviation, MC = Monte Carlo, bpvalue = Bayesian p–value, tau = precision

of Nigerian normal feather male chickens. Predicted relationship between body weight and some of the linear body measurements (SHL, THL, KEL, BOL, WIL and BOG) were positive and ranged from 0.272 ± 4.972 to 101.5 ± 24.56. The SHD, TAL and WIS had negative relationships with body weight with estimates ranging from –15.94 ± 12.31 to –4.608 ± 59.86. These positive regression coefficients obtained between body weight and aforementioned body dimensions were in tandem with reports of Olowofeso (2009) and Ojedapo et al. (2012) in their studies with chickens. However, different results were obtained when weighted multiple regression was used. This might not be unconnected with assumption of unequal variance in weighted multiple regression. Hence, when using classical analysis or unweighted multiple regression in fitting linear body measurements of chickens, independent and homoscedasticity assumptions should be tested.

Among body dimensions that had positive regression coefficient estimates in unweighted multiple regression, only BOG and TAL estimates changed to negative values (Table 3).

Table 3
Weighted multiple regression relating body weight to linear body measurements of Nigerian normal feather male chickens

Parameters	Reg. coeff.	sd	MC error	95% credible interval
Intercept	5.255	19.06	1.287	–32.32 to 18.66
Shank length	0.08307	1.029	0.06921	–2.23 to 0.7437
Shank diameter	0.3074	2.159	0.1457	–3.827 to 7.781
Thigh length	0.1064	0.6493	0.04397	–0.5162 to 2.43
Keel length	0.2202	1.019	0.06928	–0.7413 to 4.639
Body length	0.006364	0.1477	0.009577	–0.1521 to 0.3172
Tail length	–0.00844	0.1013	0.004861	–0.1968 to 0.07519
Wing length	–0.08284	1.151	0.07846	–0.7844 to 1.613
Wing span	–0.02699	0.2511	0.01705	–0.805 to 0.2936
Breast girth	–0.01974	0.2281	0.0151	–0.7115 to 0.2404
bpvalue	0.5726	0.4947	0.003373	0 to 1
tau	172.2	88.79	5.759	0.01597 to 282.9

Reg. coeff = Regression coefficient, sd = standard deviation, MC = Monte Carlo, bpvalue = Bayesian p–value, tau = precision

The unweighted multiple regression function fitted for body weight and linear body measurements in Nigerian normal feather female chicken is shown in Table 4.

Apart from TAL, WIS and BOG, all other body dimensions had positive regression coefficients in a range of 2.68 ± 3.463 to 55.2 ± 22.64. Meanwhile, regression coefficients of body dimensions in weighted regression were positive values except in TAL (Table 5).

These results obtained for weighted regression was in agreement with the studies of Adeleke et al. (2004) and Ganiyu et al.

Table 4
Unweighted multiple regression relating body weight to linear body measurements of Nigerian normal feather female chickens

Parameter	Reg. coeff.	sd	MC error	97.5% Credible interval
Intercept	–152.5	100.6	3.641	–346 to 43.74
Shank length	55.2	22.64	1.271	11.15 to 99.93
Shank diameter	18.61	53.59	3.164	–88.38 to 122.3
Thigh length	17.44	14.83	0.8413	–11.43 to 47.04
Keel length	37.66	22.41	1.333	–6.259 to 84.16
Body length	12.45	4.894	0.2947	3.399 to 23.52
Tail length	–6.162	8.011	0.3835	–21.75 to 8.965
Wing length	–5.81	9.03	0.5095	–25.17 to 10.59
Wing span	2.68	3.463	0.2069	–4.284 to 9.247
Breast girth	–17.41	10.25	0.6243	–37.27 to 1.718
tau	$3.0(10^{-5})$	$3.8(10^{-6})$	$5.37(10^{-8})$	2.30 to $3.79(10^{-5})$
bpvalue	0.4331	0.4955	0.002466	0 to 1

Reg. coeff = Regression coefficient, sd = standard deviation, MC = Monte Carlo, bpvalue = Bayesian p–value, tau = precision

Table 5
Weighted multiple regression relating body weight to linear body measurements of Nigerian normal feather female chickens

Parameter	Reg. coeff.	sd	MC error	97.50% Credible interval
Intercept	3.727	2.409	0.1623	–3.124 to 5.376
Shank length	0.03244	0.07272	0.004708	–0.1234 to 0.08932
Shank diameter	0.1414	0.183	0.01218	–0.06368 to 0.2343
Thigh length	0.01093	0.0567	0.003721	–0.03733 to 0.05468
Keel length	0.05681	0.1897	0.01288	–0.1431 to 0.1258
Body length	0.01651	0.03406	0.00229	–0.01351 to 0.0824
Tail length	$-3.67(10^{-5})$	0.01819	0.00112	–0.01331 to 0.0248
Wing length	0.01736	0.04831	0.003209	–0.02702 to 0.1342
Wing span	0.002782	0.01702	0.001141	–0.01108 to 0.02338
Breast girth	$5.19(10^{-4})$	0.1047	0.007116	–0.02948 to 0.1135
bpvalue	0.4852	0.4998	0.006023	0 to 1
tau	74.37	21.1	1.295	1.22 to 100.3

Reg. coeff = Regression coefficient, sd = standard deviation, MC = Monte Carlo, bpvalue = Bayesian p–value, tau = precision

(2016). Adeleke et al. (2004) reported the estimation of body weight of crossbred egg–type chickens from linear body measurements while Ganiyu et al. (2016) reported regression coefficients of linear body measurements for only BOL, KEL, SHL, THL and WL in Anak White broiler chickens. The 95% credible interval for the

estimates suggested that only for SHL and KEL were positive relationships between the body weight and body dimension.

The Monte Carlo (MC) errors measure the variation of the mean of the regression coefficients due to sample simulation. Lower MC errors in all the models compared to corresponding estimated standard deviation implied that estimated regression coefficients were estimated with high precision regardless of violation of regression assumption. However, estimated precision (tau) values (172.20, 74.37) for male and female chickens respectively under weighted regression model were higher than estimated tau (3.30×10^{-5}) for unweighted regression model in both sexes. This indicated better estimation accuracy of coefficient parameters under weighted models over unweighted models.

Goodness of fit of our models was assessed using Bayesian p–value, Deviance information criterion (DIC) and graph of residuals against predicted values under each model. A fitting model with a Bayesian p–value near 0.5 and value close to 0 or close to 1 suggests doubtful fit of the model (Marc, 2010). The Bayesian p–value (0.502) for unweighted regression model for male chicken was closed to 0.5 compared to its weighted counterpart (0.573). This implied that weighted regression model fitted relationship between body weight and linear body measurements in Nigerian normal feather male chicken compared to unweighted regression model. However, a different result was obtained in female chicken. Weighted multiple regression Bayesian p–value (0.4852) was closed to 0.5 compared to unweighted Bayesian p–value (0.433).

Using DIC value, Ioannis (2009) stated that the lower the DIC value, the better the fitted model. Unweighted regression model for male chicken had higher DIC value (1260.120) compared to weighted regression model (–235.002). Similar results was obtained in female chicken, with DIC value (1840.760) for unweighted regression model higher than DIC value (–201.184) for weighted regression.

Figures 1 and 3 showed the plot of residuals against the predicted values of body weight using unweighted regression model for male and female chickens respectively. The scatter plot of the residuals scattered around zero line. This implied that the variances were heteroscedastic (i.e. variances of the error terms are not equal). This implied that the estimates of the regression parameters and its standard deviation estimates were potentially biased.

Graph of residuals against predicted values of body weight of male and female chickens in weighted regression model is shown in Figures 2 and 4 respectively. It showed that the variances were homoscedastic (i.e. variances of the error terms are equal) because the residuals roughly form a horizontal band around the zero line. Hence, there is no sign of a violation of the independent and homoscedasticity assumption under weighted regression models for both male and female chickens.

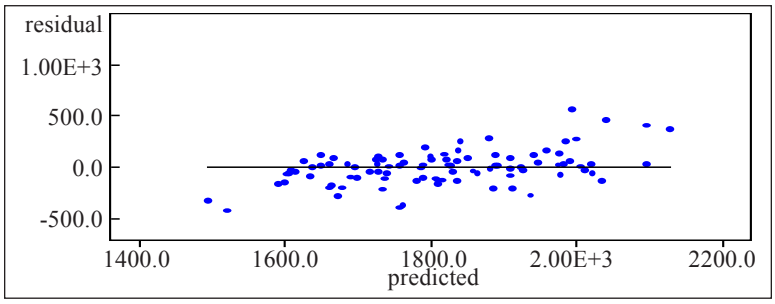

Figure 1. A plot of residual against predicted values of body weight of Nigerian normal feather male chickens using unweighted regression model

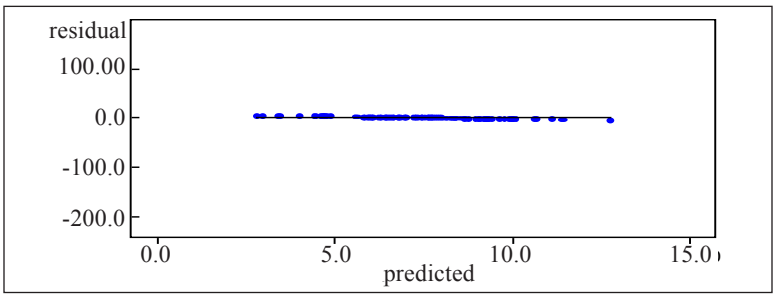

Figure 2. A plot of residual against predicted values of body weight of Nigerian normal feather male chickens using weighted regression model

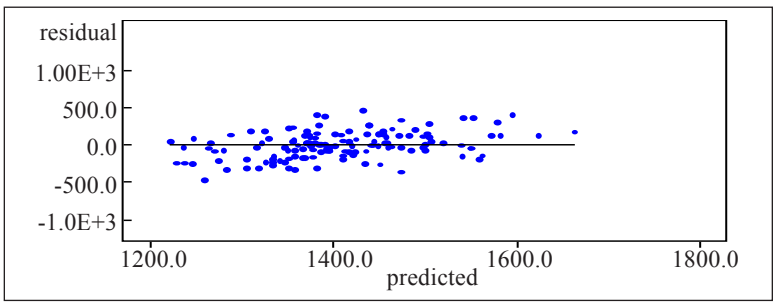

Figure 3. A plot of residual against predicted values of body weight of Nigerian normal feather female chickens using unweighted regression model

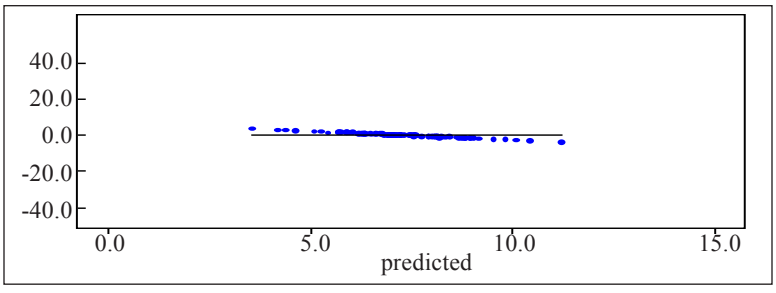

Figure 4. A plot of residual against predicted values of body weight of Nigerian normal feather female chickens using weighted regression model

CONCLUSION

The differences in DIC and Bayesian p–values and plot of residuals against predicted values of weighted and unweighted multiple regression models were sufficient for us to believe that weighted multiple regression models fitted body weight and linear body measurements data better than unweighted multiple regression models. Bayesian weighted multiple regression model is therefore recommended when fitting linear body measurements of chickens especially if prior knowledge of the parameters is available. In addition, when using unweighted linear regression in fitting linear body measurements of chickens, independent and homoscedasticity assumptions should be tested and reported in order to avoid biasness and misleading.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Effects of Animal Manures and Cutting Height on the Chemical Composition of Two *Panicum maximum* Varieties (Local and Ntchisi) Harvested at Different Stages of Growth

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ABSTRACT

An experiment was conducted to determine the effects of animal manures and cutting height on the chemical composition of two *Panicum maximum* varieties harvested at different stages of growth. The experiment was laid out as a split-split-split plot design with four manure types (cattle, swine, poultry, and control) assigned to the main plot, three stages of growth (8, 10, and 12 weeks) assigned to the sub-plots, three cutting heights (10, 15, and 20 cm above ground) allotted to the sub-sub-plot, and *P. maximum* varieties (Local and Ntchisi) allotted to the sub-sub-sub-plot with three replicates. Results showed that swine and cattle manure fertilized grasses recorded ($P<0.05$) higher crude protein (CP) content than the unfertilized and poultry manure fertilized grasses. The grasses defoliated at 8 weeks recorded higher CP (10.37%), ether extract (8.77%) and ash (9.60%) compared to those harvested at 10 and 12 weeks respectively. Ntchisi variety recorded higher ether extract (8.94%) and ash (9.58%) contents than the local variety. However, the neutral detergent fibre (NDF) and acid detergent fibre (ADF) of the grasses increased with advancement in the growth stage. Grasses cut at 15 cm had higher Ca (4.66 g kg^{-1}) while those cut at 20 cm recorded higher P (4.01 g kg^{-1}) concentration. The quality of the grasses

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was influenced by the interactions (manure × harvest time; manure × variety × cutting height; manure × cutting height × harvest time). Therefore, to harness the best nutritive *P. maximum* for the feeding of ruminants in Nigeria and other parts of the tropics, manure (cattle, swine, and poultry), harvest time, variety and cutting height, and their interactions are important factors to be considered.

Keywords: Animal manure, cutting height, macro-mineral, *Panicum maximum*, ruminants, stage of growth

INTRODUCTION

Panicum maximum is one of the dominant forage species in the humid and sub-humid zones of Nigeria. The grass plays a vital role in livestock production owing to its persistence, growth and quality under proper management through grazing or clipping (Olanite et al., 2014). The significance of *P. maximum* as one of the most relished feed resource by ruminants in Southwest Nigeria was the reason why it featured prominently in the early evaluation and grazing studies in the area (Ademosun, 1973; Akinyemi & Onayinka, 1982). Some of the characteristics that enhance high utilization of *P. maximum* by animals include high leaf production, particularly at the early stage of growth (Olanite et al., 2006), as well as resilience to grazing by ruminants.

Several animal manures have been reported as being suitable for soil amendment (Ewetola et al., 2016; Ojo et al., 2015) and for improving the nutritive quality of tropical plants. Swine manure, cattle dung, farmyard manure and poultry droppings are potential soil amendments for crop and pasture plants (Ahmed et al., 2012; Ojo et al., 2015; Sodeinde et al., 2009). Besides enhancing forage growth, organic manures also possess the capability to improve the physical, chemical, and microbial properties of the soil (Adesodun et al., 2005). Thus, fertilization of pastureland with organic manure sits well within the context of nutrient recycling and environmental protection. Ewetola et al. (2016) reported that application of organic manure directly to the soil helped in maintaining adequate level of organic matter, which was a critical component of soil fertility and productivity.

Several species of *P. maximum* have been subjected to different evaluation studies in southwest Nigeria over time (Bamikole et al., 2004; Ezenwa & Aken'Ova, 1998; Olanite et al., 2006). These studies have shown a strong relationship between extended cutting intervals and stage of growth with declined nutritive quality, but without manure application and consideration of different varieties. In contrast, short cutting intervals enhance nutritive quality of the grass. Options therefore vary on the cutting interval that would be consistent with maintaining high nutritive quality of *P. maximum.*

Panicum maximum Ntchisi is rarely used as a major forage in commercial livestock production in south western Nigeria. This is because it has to be established and managed, unlike the local variety which

is freely available for use by livestock farmers. The need to establish pastures to cater for the increasing demand for animal products and ever increasing human population in the derived savannah zone of Nigeria will soon become unavoidable (Olanite et al., 2006). This will ultimately require research findings of this kind for management purpose. To this end - the utilization of *P. maximum* by farmers in Nigeria requires improved information on the relative performance of varieties, with regard to how they respond in terms of herbage quality, including nutrient content, to different types of organic manure, and to harvesting at different growth stages. The present study aims at investigating the effects of animal manures and cutting height on the chemical composition of Ntchisi and the locally available variety of *P. maximum,* harvested at different stages of growth with a view to make recommendations to commercial farmers on the nutritive value of the grass species; for livestock feeding which could further facilitate their use for feeding ruminants in Nigeria and other tropical regions.

MATERIALS AND METHODS

Experimental Site and Land Preparation

The experiment was conducted at the Directorate of University Farms, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria ($7^{o}58'$N; $3^{o}2^{o}$E) in the humid savannah agro-ecological zone. The area has a mean annual rainfall of 1230 mm with a bimodal distribution pattern of rainfall that attains peak in June/July and September/ October with major dry season between November and March. The experimental area measured 656 m^2 (41 × 16 m), and was part of a virgin land which was opened for research purpose using machinery. The land was ploughed twice, followed by harrowing to smoothen the soil surface. The lumps of soil left after ploughing were also broken down through harrowing to ensure a finer finish, and good soil structure suitable for plant growth. Soil samples were collected from the experimental site at a depth of 0-15 cm (Table 1) to determine the physico-chemical characteristics of the soil prior to application of manure.

Table 1

Physico-chemical characteristics of the composite soil samples taken at 0-15 cm depth from the experimental site before planting

Chemical Properties	Values
pH	7.03
Total nitrogen (%)	0.11
Organic carbon (%)	1.29
C:N ratio	28.38
Available P (mg kg^{-1})	53.87
Acidity (cmol kg^{-1})	0.13
CEC	1.79

Table 1 *(Continue)*

Chemical Properties	Values
Exchangeable cations(cmol kg^{-1})	
Sodium (Na)	0.80
Potassium (K)	0.20
Calcium (Ca)	2.77
Magnesium (Mg)	2.72
Particle Size	
Sand (%)	77.93
Silt (%)	17.33
Clay (%)	4.73

Manure Collection, Analysis and Application

The manures used were: cattle, swine, poultry and control. The cattle and swine manures were collected at the College of Animal Science and Livestock Production (COLANIM) farm, poultry manure was sourced at Livelihood Support and Development Centre (SLIDEN Africa) farm located at Alabata Road. Samples of all the manures were collected and taken to the laboratory for determination of their nutrient content prior to application (Table 2). The manures were applied at 120 kg N per hectare. These manures were manually incorporated into the soil for proper and efficient mineralization. The field was left for two weeks after manure application before planting of the grasses.

Table 2
Nutrient composition of animal manures

Parameters	**Cattle**	**Swine**	**Poultry**
N (g kg^{-1})	15.6	16.9	30.2
P (g kg^{-1})	6.9	6.3	10.6
K (g kg^{-1})	7.3	7.6	10.3
Ca (g kg^{-1})	21.2	31.6	37.2
Mg (g kg^{-1})	11.7	19.2	17.3
Na (g kg^{-1})	1.1	1.6	2.1
Fe (mg kg^{-1})	614.6	650.7	630.9
Zn (mg kg^{-1})	54.8	81.2	75.4
Cu (mg kg^{-1})	29.1	27.3	32.7
Mn (mg kg^{-1})	321.9	260.3	217.9

Sourcing of Planting Materials and Planting

The two *P. maximum* varieties – a naturalized variety commonly referred to as local Panic (Olanite et al., 2006) and the Ntchisi variety which originated from East Africa (Skerman, 1977) were sourced from an open land and the introduction plot of the Department of Pasture and Range Management. The planting materials were separated into crown splits before planting at a spacing of 0.5 × 0.5 m. The inter and intra row spaces within the plots, as well as 2 m from the boundary of the plots were kept weed free throughout the research period in order to prevent weed invasion, as well as other forms of interference.

Treatments, Experimental Design and Plot Layout

The experiment comprised four factors which were four manure types (cattle, poultry, swine and control i.e. no application of manure), two *P. maximum* varieties (Local and Ntchisi); three stages of growth (8, 10, and 12 weeks) and three cutting heights (10 cm, 15 cm and 20 cm) respectively. The manure types were assigned to the main plots (41 × 4 m), the stages of growth assigned to the sub plots (4.5 m × 4 m), cutting heights were assigned to the sub-sub plots (4.5 m × 1 m) and the grass varieties were allotted to the sub-sub-sub plots (1 m × 1 m). The treatments were replicated thrice giving a total of 216 plots, with each plot having a dimension of 1 m × 1 m. The space between the replicates was 2 m and 0.5 m spacing was maintained between the treatments.

Sample Collection and Preparation

Grass samples were harvested at 8, 10 and 12 weeks after planting while clipping was done at 10, 15, and 20 cm from the ground level. Sub samples were taken, packed and oven dried at 65^{o}C to a constant weight and subsequently milled to pass 1mm sieve and stored for analysis.

Chemical Analysis

The content of dry matter (DM), crude protein (CP), ether extract (EE i.e. fat) and ash (inorganic minerals) were determined according to Association of Official Analytical Chemists (AOAC) (2010), while the neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), hemicellulose (HEM), and cellulose (CELL) were determined according to the procedure of Van Soest et al. (1991).

Mineral Composition Analysis. The milled samples of the grasses were used for mineral analysis of Calcium (Ca), Potassium (K), Phosphorus (P), and Magnesium (Mg). The concentration of K was estimated with a flame photometer while the concentrations of Ca, P and Mg were determined using atomic absorption spectrophotometry (Fritz & Schenk, 1979) after wet digestion in 15 ml nitric acid and 5 ml hydrochloric acid.

Statistical Analysis

All data collected were subjected to four-way analysis of variance (ANOVA) using the split-split-split plot design in R Statistics (R Core Team, 2015). Significant differences between individual means were separated using Duncan's Multiple Range Test.

RESULTS

Manure type ($P<0.05$) affected the proximate composition of the two grass varieties except for dry matter (DM) content (Table 3). The crude protein (CP) content of the grasses ($P<0.05$) ranged from 8.83% in the unfertilized grasses to 10.77% in swine manure fertilized grasses. Cattle manure fertilized grasses and those fertilized with swine manure obtained higher statistically similar values (10.92% and 10.25%) for ether extract (EE), and the lowest (5.92%) was recorded for the unfertilized grasses. The values recorded for ash ranged from 8.52% for poultry fertilized grasses to 10.50% in the unfertilized grasses. However, the ash values observed for swine manure and poultry manure fertilized grasses were statistically similar.

The DM and EE content of the grasses were ($P<0.05$) influenced by cutting height. The grasses harvested at 10 cm and 15 cm height had statistically similar values (94.27% and 94.20%) for DM, while those harvested at 20 cm recorded the lowest value (92.54%). The grasses cut at 10 cm height recorded higher EE (8.77%) than those cut at 15 cm height above the ground.

Grasses harvested at 8 weeks recorded the highest CP and EE values (10.37 % and 9.13 %) respectively, while the least values (9.48 % and 7.67 %) were observed for those harvested at 12 weeks of age, but statistically similar to those harvested at 10 weeks for CP. In contrast, grasses harvested at 8 and 12 weeks recorded the highest value (9.54 %) for ash content, while those clipped at 10 weeks had the least value (9.08 %) for the same parameter.

Table 3

Effect of manure type and cutting height on the proximate composition (%) of two P. maximum *varieties (local and Ntchisi) harvested at different stages of growth*

Factors	DM	CP	EE	ASH
Manure				
Cattle	92.97	10.60^{a}	10.92^{a}	9.94^{b}
Swine	93.77	10.77^{a}	10.25^{a}	8.58^{c}
Poultry	94.08	8.97^{b}	6.56^{b}	8.52^{c}
Control	93.86	8.83^{b}	5.92^{b}	10.50^{a}
SEM	0.45	0.29	0.33	0.21

Table 3 *(Continue)*

Factors	DM	CP	EE	ASH
Cutting Height				
10cm	94.27[a]	10.06	8.77[a]	9.60
15cm	94.20[a]	9.82	8.04[b]	9.31
20cm	92.54[b]	9.51	8.42[ab]	9.25
SEM	0.36	0.28	0.39	0.21
Harvest Time				
8 weeks	93.85	10.37[a]	9.13[a]	9.54[a]
10 weeks	93.60	9.53[b]	8.44[b]	9.08[b]
12 weeks	93.56	9.48[b]	7.67[c]	9.54[a]
SEM	0.40	0.27	0.38	0.20
Variety				
P. maximum (Local)	93.88	9.70	7.88[b]	9.19[b]
P. maximum (Ntchisi)	93.45	9.88	8.94[a]	9.58[a]
SEM	0.32	0.23	0.31	0.17
Cutting H × Har Time	0.9749	0.0796	0.0557	0.0013
Manure × Cutting H	0.8104	0.6484	0.0008	<0.0001
Variety × Cutting H	0.4426	0.2834	0.1391	0.9438
Manure × Har Time	<.0001	0.2520	<0.0001	<.0001
Variety × Har Time	0.0014	0.4100	0.0059	0.0357
Manure × Variety	0.3727	0.0172	0.4728	<0.0001
Manure × Cut H ×Har T	<0.0001	0.0056	0.0815	0.0020
Var × Cut H × Har T	0.1369	0.0017	0.4757	0.0965
Manure ×Var × Cut H	0.0066	0.0691	0.0243	0.1003
Manure ×Var × Har T	0.3098	0.6291	0.1137	0.0255

[a,b,c] means in the same column with different superscripts are significantly different (P<0.05) SEM = Standard error of mean, DM = Dry matter, CP = Crude protein, EE = Ether extract, ASH = Ash. P values are reported for the interactions

The influence of variety was (P<0.05) pronounced only in the EE and ash content of the grasses. *P. maximum* Ntchisi recorded (P<0.05) higher values (8.94% and 9.58%) for ether extract and ash, while the local variety recorded the lowest values (7.88 % and 9.19 %).

The DM content of the grasses was significantly affected by manure × harvest time, variety × harvest time, manure ×

cutting height × harvest time and manure × variety × cutting height. The CP content was affected by manure × variety, manure × cutting height × harvest time, and variety × cutting height × harvest time. Cutting height × harvest time, manure × cutting height, manure × harvest time, variety × harvest time, and manure × variety × cutting height had significant effect on the EE content of the grasses.

The values recorded for acid detergent fibre (ADF) ranged from 47.61% for cattle manure fertilized grasses to 57.61% in the unfertilized grasses (Table 4). Nonetheless, grasses fertilized with swine and poultry manure recorded statistically similar values. Swine manure fertilized grasses had the highest value (14.44 %) for acid detergent lignin (ADL) while cattle (12.22%) and poultry (11.67%) manure, as

Table 4

Effect of manure type and cutting height on the fibre composition (%) of two P. maximum *varieties (local and Ntchisi) harvested at different stages of growth*

Factors	NDF	ADF	ADL	HEM	CELL
Manure Types					
Cattle	62.00	47.61^{c}	12.22^{b}	14.38^{d}	45.39^{a}
Swine	75.27	55.78^{b}	14.44^{a}	19.50^{b}	41.33^{b}
Poultry	76.06	52.50^{b}	11.67^{b}	23.56^{a}	40.83^{b}
Control	74.33	57.61^{a}	12.17^{b}	16.72^{c}	45.44^{a}
SEM	0.70	0.80	0.55	0.90	0.90
Cutting Height					
10 cm	64.21	45.92	13.13	18.29	42.79
15 cm	74.29	56.25	12.21	18.04	44.04
20 cm	74.75	55.46	12.54	19.29	42.92
SEM	0.68	0.74	0.49	0.88	0.83
Harvest Time					
8 weeks	65.62^{b}	47.79^{b}	12.25	17.83	45.54^{a}
10 weeks	73.63^{ab}	55.08^{a}	12.33	18.54	42.75^{b}
12 weeks	74.00^{a}	54.75^{a}	13.29	19.25	41.45^{b}
SEM	0.68	`	0.49	0.88	0.80

Table 4 *(Continue)*

Factors	NDF	ADF	ADL	HEM	CELL
Variety					
P. maximum (Local)	65.44[b]	47.63[b]	13.89	17.80	45.02[a]
P. maximum (Ntchisi)	73.39[a]	54.11[a]	13.45	19.27	41.47[b]
SEM	0.55	0.58	0.40	0.72	0.66
Cutting H × Har Time	0.9454	0.1217	0.2276	0.1641	0.9100
Manure × Cutting H	0.2756	0.1730	0.5257	0,0868	0.2438
Variety × Cutting H	0.1057	0.8118	0.3008	0.0723	0.5957
Manure × Har Time	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Variety × Har Time	0.8140	0.0241	0.0085	0.1087	0.9019
Manure × Variety	0.2380	0.1725	0.2489	0.1296	0.4573
Manu × Cut H × Har T	0.4907	0.0037	0.0066	0.0098	0.1689
Var × Cut H × Har T	0.4395	0.0498	0.8067	0.0307	0.4621
Maunre × Var × Cut H	0.8226	0.3123	0.0044	0.3143	0.1958
Manure × Var × Har T	0.4373	0.0438	0.1328	0.0672	0.2794

[a,b,c]: means in the same column with different superscripts were significantly different, (P<0.05) SEM = Standard error of mean, NDF =Neutral detergent fibre, ADF = Acid detergent fibre, ADL = Acid detergent lignin HEM = Hemicellulose, CELL = Cellulose. P values are reported for the interactions

well as the unfertilized grasses (12.17%) recorded values that were statistically at par. Meanwhile, grasses fertilized with poultry manure obtained the highest value (23.56 %) for hemicellulose (HEM), while those fertilized with cattle manure had the lowest value (14.38 %). In contrast, the unfertilized grasses and those amended with cattle manure had similar values (45.44 % and 45.39%) for cellulose (CELL) while grasses fertilized with poultry and swine manure recorded significantly lower values (40.83% and 41.33%). Cutting height did not influence the fibre composition of the grasses significantly (P>0.05). However, stage of growth (P<0.05) affected the neutral detergent fibre (NDF), ADF, and CELL content of the grasses. The grasses harvested at 12 weeks recorded higher NDF (74.00%) than those harvested at 8 weeks of growth (65.62%). Similarly, the values recorded for ADF was (P<0.05) higher for the grasses harvested at 10 and 12 weeks (55.08% and 54.75%), and the least (47.79 %) was observed for those harvested at 8 weeks. The grasses harvested at 8 weeks recorded the highest value (45.54%) for CELL content while those harvested at 10 and 12 weeks had similar values. The effect of variety was significant (P<0.05) on the NDF, ADF and CELL content of the grasses. *P. maximum* Ntchisi recorded higher values

(73.39 % and 54.11%) for NDF and ADF, and the local variety was observed for higher CELL content.

The interaction between manure × harvest time was ($P<0.05$) for all the fibre component of the grasses. Other significant ($P<0.05$) interactions are: variety × harvest time ($P=0.0241$) for ADF, manure × cutting height × harvest time ($P=0.0037$) for ADF, variety × cutting height × harvest time ($P=0.0498$) for ADF, manure × variety × harvest time ($P=0.0438$) for ADF, variety × harvest time ($P=0.0085$) for ADL, manure × cutting height × harvest time ($P=0.0066$) for ADL, manure × variety × cutting height ($P=0.0044$) for ADL and variety × cutting height × harvest time ($P=0.0307$) for HEM.

The unfertilized grasses recorded higher potassium (K) concentration (35.01 g kg^{-1}) while the lowest concentration was observed for poultry manure fertilized grasses (Table 5). The grasses fertilized with cattle and swine manure, as well as the unfertilized grasses recorded ($P<0.05$) higher values for calcium (Ca) (4.90 g kg^{-1}, 4.60 g kg^{-1}, and 4.37 g kg^{-1}) and the least value was recorded for poultry manure fertilized grasses (3.22 g kg^{-1}). With regard to cutting height, phosphorus (P) and Ca content of the grasses were ($P<0.05$) affected. The grasses harvested at 20 cm height recorded higher P than those harvested at 10 cm height above ground. In contrast, Ca concentration was higher for the grasses cut at 15 cm height (4.66 g kg^{-1}) than those defoliated at 10 cm height (3.74 g kg^{-1}).

Table 5

Effect of manure type and cutting height on the macro-mineral concentration (g kg^{-1}) of two P. maximum *varieties (local and Ntchisi) harvested at different stages of growth*

	Phosphorus	Potassium	Magnesium	Calcium
Factors		g kg^{-1}		
Manure Types				
Cattle	4.28	32.75^{ab}	2.03	4.90^{a}
Swine	4.34	32.52^{ab}	2.05	4.61^{a}
Poultry	3.73	31.61^{b}	1.69	3.22^{b}
Control	4.04	35.01^{a}	1.91	4.37^{a}
SEM	0.03	0.97	0.13	0.04
Cutting Height				
10 cm	3.73^{b}	33.73	1.80	3.74^{b}
15 cm	4.01^{ab}	32.66	1.97	4.66^{a}
20 cm	4.55^{a}	32.52	1.99	4.42^{ab}
SEM	0.02	0.88	0.11	0.03

Table 5 *(Continue)*

	Phosphorus	Potassium	Magnesium	Calcium
Factors		g kg^{-1}		
Stage of Growth				
8 weeks	3.44^{b}	33.39	2.18^{a}	5.13^{a}
10 weeks	4.90^{a}	33.80	1.51^{b}	2.13^{b}
12 weeks	3.95^{b}	31.71	2.07^{a}	5.56^{a}
SEM	0.02	0.71	0.09	0.03
Varieties				
Local	3.90	31.98^{b}	1.88	4.18
Ntchisi	4.30	33.96^{a}	1.95	4.37
SEM	0.02	0.85	0.11	0.03
Cutting H × Har Time	<0.0001	0.3300	<0.0001	<0.0001
Manure × Cutting H	0.1650	0.1860	0.1470	0.0053
Variety × Cutting H	0.0223	0.0133	0.3710	0.2890
Manure × Har Time	<0.0001	<0.0001	<0.0001	<0.0001
Variety × Har Time	<0.0001	<0.0001	0.0002	<0.0001
Manure × Variety	0.264	0.0007	0.2580	0.0074
Manu × Cut H × Har T	<0.0001	<0.0001	0.0018	<0.0001
Var × Cut H × Har T	<0.0001	<0.0001	0.0060	<.0001
Maunre × Var × Cut H	0.0090	0.0027	0.2180	0.0044
Manure × Var × Har T	<0.0001	<0.0001	<0.0001	<0.0001

^{a,b,c}: means in the same column with different superscripts were significantly different ($p<0.05$)
SEM=Standard Error of Mean. P values are reported for the interactions.

All the macro minerals investigated were influenced by stage of growth except for K. The highest P concentration was observed for the grasses harvested at 10 weeks (4.90 g kg-^{1}). However, P concentration recorded for the grasses defoliated at 8 and 10 weeks were statistically similar. Magnesium (Mg) concentration observed for the grasses harvested at 8 and 12 weeks were statistically at par and the lowest was noted for those cut at 10 weeks of growth (1.51 g kg^{-1}). Higher ($P<0.05$) Ca content was recorded for the grasses clipped at 8 and 12 weeks, with the least observed for those harvested at 10 weeks. The effect of variety was only significant on the K content of the grasses with Ntchisi variety recording higher concentration (33.96 g kg^{-1}) compared to that (31.98 g kg^{-1}) observed for the local variety.

There were significant interactions between manure × harvest time, variety × harvest time, and manure × variety × harvest

time for all the macro mineral concentration of the grasses. With the exception of K, the effect of cutting height × harvest time was observed on all other mineral content of the grasses. Phosphorus, K, and Ca were significantly affected by manure × cutting height × harvest time and variety × cutting height × harvest time.

DISCUSSION

The result from this study revealed that the CP content of the grasses under the influence of manure type was above the minimum requirement for maintenance of ruminant animals. These values were also above the minimum range of 6.50 – 8.00% prescribed for optimal performance of tropical ruminant animals (Minson, 1981). The highest CP recorded for the grasses fertilized with cattle and swine manure might be linked to high nitrogen content in the manure (Chantigny et al., 2007), and this suggests that manure mineralization takes place at different rates relative to the donor animal. Thus, our result further ascertains that swine manure promotes high accumulation of CP and this corroborates earlier reports (Ojo et al., 2013; Olanite et al., 2006).

The CP content of the grasses was not influenced by cutting height and this agrees with the report by Wijitphan et al. (2009) on the CP of King Napier grass investigated in Thailand. Higher CP was noted for grasses harvested at 8 weeks of growth than for those harvested at 10 and 12 weeks with similar CP contents. The observed decline in the CP content of the grasses could be attributed to decrease in leaf stem ratio and increase in cell wall content of the grasses with advancement in growth stage. The range of values recorded by the two varieties of *P. maximum* investigated in this study showed that *P. maximum* Ntchisi recorded higher EE and ash content values than the local variety, suggesting that Ntchisi variety could provide ruminants with more energy and total inorganic mineral content. This could be ascribed to the fact that Ntchisi is an improved variety over the local panic, a naturalized variety (Olanite et al., 2006).

The interaction effect of variety × cutting height × harvest time and manure × variety observed on the CP content of the grasses agrees with the report by Assefa and Ledin (2001) and Chaves et al. (2006) that fertilizer, harvest time, and variety rank among the factors that determined the quality of forage. A similar result was reported by Mohajer et al. (2012) when three varieties of common millet (*Panicum miliaceum*) were harvested at three phonological growth stages (booting, milky, and seed maturity). However, proportional changes in plant nutrient composition is an important consideration when pastures are managed for the production of high nutrient intake for ruminant animals (Chaves et al., 2006).

Not only do the NDF and ADF fractions of the grasses differ at 8 and 12 weeks of age but also increased, and this could be premised on increase in the cell wall content and a decrease in the leaf-stem ratio of the grasses which consequently lead to accumulation of lignin (Moore & Jung, 2001). From this point of view, the grasses harvested at 8 weeks of growth are of higher

nutritional value which could promote better intake. Values recorded for ADF under the influence of manure type were slightly above the range of 29.78%-32.52% reported by Okukenu et al. (2017) when nine varieties of *Pennisetum purpureum* were evaluated in the derived savanna of Nigeria. The observed disparity could be attributed to difference in manure application rate. Moreover, the low level of lignin recorded for the grasses as affected by manure type suggests that the cellulose content of the grasses would be moderately digestible, since increased lignin in forages limits the digestibility of cellulose (Hatfield et al., 1999; Moore & Hatfield, 1994). The ADF and ADL values observed were slightly higher than those reported by Ojo et al. (2015) when similar manure types were applied to Pennisetum hybrid.

Compared with our results, Fernendes et al. (2014) recorded slightly lower ADF values for *P. maximum* genotypes fertilized with dolomitic lime in the Brazilian savannah. The observed difference could be partly due to the use of different soil amendments in the former and present study. Doubtless, higher NDF content of the local variety was due to the presence of higher proportion of stem material in the forage, and this may not have much interference with the digestibility of the plant at a younger age; since tender stems could be as digestible as the leaves. More importantly, the advancement in the age of the plants also affects the leaves as they become more fibrous and less digestible. The effect of cutting height was not observed on the fibre fractions of the grasses. Earlier reports (Ojo et al., 2015, Zetina-Cordoba et al., 2013) showed that nutritive quality declined as the cutting interval extends as depicted by the CP, NDF and ADF components of the grasses. However, there was a slight deviation in the result obtained in this study as the NDF and ADF fluctuate between 8 and 10 weeks of growth probably due to alterations in leaf: stem ratio.

The effect of variety influenced all the fibre fractions of the grasses except for ADL and HEM. The values observed for CELL content of the grasses as affected by stage of growth were slightly higher than (25.65%-39.26%) reported by Ojo et al. (2013) for *P. maximum*. The observed disparity could be hinged on the application of (N: P: K 15:15:15) at 200 kg N/ha in the former study, as well as the time of application. Moreover, grasses harvested at 12 weeks of growth recorded higher bulk of fibre (NDF and ADF) , and this implies high lignification as a result of the development of more structural components which makes the materials undesirable for feeding ruminants at high maturity stage. This would most likely result in decreased digestibility and acceptance of such forage materials by ruminants.

Johnson et al. (2001) reported a significant interaction effect of N fertilization and harvest date for NDF content of three tropical forage species (Bahia grass, Bermuda grass, and Star grass), while Mohajer et al. (2012) reported non-significant effect of variety × growth stage for ADF content of three varieties

of common millet (*P. miliaceum*). Our data agrees with the former report, but contradicts the latter. However, in this study, all the fibre fractions of the grasses were significantly influenced by the interaction of manure × harvest time and this is not in consonance with the report by Anderson et al. (2013) who reported no variation in the CELL, HEM, and lignin content of switch grass under the influence of harvest time × nitrogen sources. This could be attributed to differences in forage species, nitrogen sources, as well as application rate.

The level of minerals in plants depends on the interactions among a number of factors including soil type, plant species, dry matter yield, grazing management and climate (Khan et al., 2005). However, Olanite et al. (2006) submitted that stage of maturity was undoubtedly a critical factor that impacted forage mineral composition. The values recorded for Ca content of the grasses as influenced by manure type, cutting height, and stage of growth in this study is suitable to meet the recommended requirement for different ruminant animals which ranges from 1.8 to 8.2 g kg^{-1} as reported by McDowell (1992). Reuter and Robinson (1997) also suggested Ca requirement for maintenance of growing and lactating sheep to be 1.2-2.6 g kg^{-1} which the grasses evaluated could provide. McDowell and Valle (2000) reported that inadequate intake of Ca was capable of causing weakened bones, slow growth, low milk production and tetany (convulsions) in severe deficiencies. Following the report of McDowell (1992), the grasses under study have suitable Mg content under the influence of growth stage as the requirements for different ruminant animals were put between 1-2 g kg^{-1}. Although 2 g Mg kg^{-1} DM is adequate to meet the Mg requirements in most situations, cows and ewes near parturition may need extra Mg (10 to 30 g Mg/cow/day or 2 to 3 g Mg/ewe/day) relative to their physiological status (Mayland, 1999). The Mg concentration of the grasses in this study as influenced by stage of growth is in agreement with the findings by Cheema et al. (2011) with regard to ruminant requirement. Low concentration of Mg in grasses can lead to hypomagnesemia (grass tetany) which is probably the most important metabolic problem in ruminants and is characterized by low blood plasma Mg concentrations (<0.4 mmol L") and most assuredly by low urinary Mg concentrations (< 0.8 mmol L") (Mayland, 1999). The grasses analysed in this study have the ability to meet up with this requirement.

Minimum critical levels of K for animals may be in the range of 5 to 10 g kg^{-1} DM. However, it has been suggested that ruminants with high producing ability in terms of milk and other animal products may require K level above 10 g kg^{-1} under stress situation particularly heat stress (McDowell, 1985), which the varieties of *P. maximum* evaluated in this study could supply. The high K content in these grasses ascertain water balance and osmotic pressure regulation, acid-base balance, and muscle contraction for ruminants (Olanite et al., 2018). There is paucity of information

on K deficiency for ruminants grazing exclusively on forages. Meanwhile, Farhad (2012) reported that high potassium forages and low sodium chloride diets appeared to contribute more to reproductive losses in herbivores. The authors concluded that excess K in forages lead to deficiency of other minerals, with attendant suppression of immunity.

The values recorded for P content of the grasses as influenced by cutting height and stage of growth in this study is suitable to meet the recommended requirement for different ruminant animals which ranges from 1.8 to 4.8 g kg^{-1} as reported by McDowell (1992). For grazing livestock, the most prevalent mineral-element deficiency throughout the world are those related to P (Underwood, 1981), with higher occurrence in the tropics. The P status of forages could vary widely due to a number of factors including cutting height and stage of growth as observed in our data.

CONCLUSION

The general indication from this study affirms that *P. maximum* Ntchisi is undoubtedly superior to the local variety in terms of chemical composition, particularly when fertilized with swine or cattle manure, clipped at 10 cm above the ground, and at 8 weeks of growth. The nutritive quality of *P. maximum* was influenced by the interactions (manure × harvest time; manure × variety × cutting height; manure × cutting height × harvest time). *Panicum maximum* Ntchisi can meet the high energy required for growth and maintenance by ruminants, with sufficient inorganic minerals for enhanced metabolic and other body functions compared to the local variety. Hence, there is benefit in the continuous use of *P. maximum* varieties for the feeding of ruminants in Nigeria and other tropical regions.

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Growth and Fruit Physico-chemical Characteristics of 'MD-2' Pineapple (*Ananas comosus* L.) at Varying Seedbed Configurations

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ABSTRACT

Seedbed configuration inevitably affects the growth and development of fruit crops in a way that canopy overlaps which might lead to intraspecific competition. Hence, this study was conducted to evaluate the effects of varying seedbed configurations on growth and fruit physico-chemical characteristics of 'MD-2' pineapple. The experiment was arranged in a randomized complete block design (RCBD) with four treatments and three replications. Seedbed configurations (25, 28, 30 and 32 seedbeds block^{-1}, respectively) with a constant of 75,000 planting density hectare^{-1} served as treatments. All plants received similar intercultural management practices employed in commercial pineapple farm. Results revealed that growth and fruit physico-chemical characteristics of 'MD-2' pineapple were comparable in all seedbed configurations used. The results indicate that 'MD-2' pineapple production is still feasible using the 25 to 32 seedbeds block^{-1} configurations with a 75,000 planting density hectare^{-1}.

Keywords: 'MD-2' pineapple, pineapple density, pineapple planting, planting configuration, seedbed per block

INRODUCTION

Pineapple is one of the major fruit crops grown in the world. Its worldwide yield increased from 15.7 million tons in 2001 to 21.6 million tons in 2011 (Genefol et al., 2017). In the Philippines, pineapple production increased by 3.0 percent during last quarter of 2017 reaching the level of 699.22 thousand metric tons (Philippine

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Statistics Authority [PSA], 2017) of which Northern Mindanao region is the major producer.

One of the major factors to consider in commercial pineapple production is seedbed configuration. This cultural management practice could significantly affect the yield and physico-chemical characteristics of fruit crops including pineapple. Several studies have been conducted using varying plant population per hectare in PR-1 67 (*Ramírez* & Gandia, 1982), Chinese Smooth Cayenne (Hung et al., 2011) and MD-2 (Genefol et al., 2017) cultivars using similar number of seedbeds block^{-1}.

Areas intended for mechanized field operations may be laid out in blocks separated by roads. The dimensions of blocks are designed to accommodate equipment and effectively accomplished the required field operations (Hepton, 2003). Each block is typically composed of raised seedbeds. If boom sprayer equipment is to be used, block width is usually twice as wide as the spray boom (Hepton, 2003). In Valencia City, Bukidnon, Philippines, the 25 double row seedbed block^{-1} with a 75,000 planting density hectare^{-1} is recommended for commercial pineapple production (T.S. Castro, personal communication, November 8, 2013). However, this configuration has closer distance between hills which might contribute to inferior plant growth, smaller fruits and/or poor fruit quality.

Hence, this study was conceptualized to evaluate the effects of varying seedbed configurations on the growth and fruit physico-chemical characteristics of 'MD-2' pineapple.

MATERIALS AND METHODS

The experiment was conducted at Mt. Kitanglad Agricultural Development Corporation (MKADC), Lurugan, Valencia City, Bukidnon, Philippines with in an elevation of 450 meters above sea level (masl). The soil was identified as Adtuyon clay. Based on laboratory analysis, soil texture was classified as clay loam. Moreover, soil pH was within the optimum range of growing pineapple. Organic matter, P, Ca and Mg were above the critical levels. Only K was found below the critical level (Table 1) for pineapple production.

Prior to the conduct of experiment, three months fallow period was employed in the particular research area. Harrowing of the experiment area was conducted twice at monthly interval. Deep plowing (mouldboard) then commenced one month after the last harrow activity. Seedbed establishment was done through the use of animal-drawn plow to attain the desired seedbed width, distance between seedbeds and walk-space distances which are some of the variations comprising each treatment (Table 3).

The experiment was laid out in a randomized complete block design (RCBD) with four treatments replicated into three. Each experiment unit had an area of 0.13 hectare with 10,000 plants. Roads were constructed (Figure 1) to separate each experiment unit. Figures 2, 3 and 4 shows the seedbed configurations employed at MKADC farm which served as the control treatment. Distances in Figures 2 and 3 were adjusted and number of seedbeds block^{-1}

(Figure 4) being modified to come up with varying number of seedbeds which served as treatments whereas maintaining the planting density per hectare to 75,000. Regardless of treatment, seedbed height was maintained at 25-30 cm (Figure 5).

Planting material used was medium sucker weighing 300 to 350 grams. Moreover, four data stations comprising a total of 800 data plants (200 data plants station^{-1}) were established within each experiment unit.

Table 1
Soil physico-chemical properties of the experiment area prior to planting

SOIL PHYSICO-CHEMICAL PROPERTY	Texture	pH	Organic Matter (%)	P	K	Ca	Mg
				----------ppm----------			
Experiment area	Clay loam	5.00	3.04	16	179	384	121
Critical level[1/]	nd	4.50-5.20	3.00	12	300	100	50

[1/]- critical levels adopted by MKADC (T.S. Castro, personal communication, November 8, 2013)

Similar cultural management practices such as rate/timing of fertilizer application, pest/disease control, and flower induction treatment were employed to all treatments after planting. Total plant nutrients (462 kg N, 143 kg P, 523 kg K, 223 kg Ca, 205 kg Mg, 24 kg Fe, 4 kg Zn, 3 kg B and 560 kg S) per hectare were applied through pre-plant application (dolomite), side dress applications (di-ammonium phosphate, ammonium sulphate, potassium sulphate and magnesium sulphate) and foliar applications (urea, iron sulphate, zinc sulphate, solubor, potassium sulphate, calcium phosphate and calcium boron) based on MKADC farm fertilization program. Flower induction treatment (Ethrel + urea) was applied at 11.5 months after planting (MAP). As a standard practice in commercial pineapple production, degreening or fruit ripening solution (Ethrel + phosphoric acid) was applied 155 days after flower induction treatment. Fruits were harvested at shell color index 2-3. In this experiment, four harvest rounds were made to clear all data fruits in each experimental unit.

Data gathered were the following:

1. Plant height- measurement of plant height was conducted at 6 months after planting (MAP), 8 MAP, 10 MAP and prior to flower induction treatment. Height (ground level to the tip of tallest leaf) of plant was measured using a measuring stick. Average plant height (APH) was computed using the formula:

$$\text{Plant height} = \frac{\sum \text{Plant height}}{\text{Number of data plants}}$$

2. Plant mass- this data was taken at 6 months after planting (MAP), 8 MAP, 10 MAP and prior to flower induction treatment. Three representative plants from the border rows were pulled-out and

weighed excluding the stump apex (below ground level). The mass of three representative plants served as baseline data in estimating the plant mass of data plants. Average plant mass was computed using the formula:

$$\text{Plant mass} = \frac{\sum \text{Plant mass}}{\text{Number of data plants}}$$

3. Fruit size distribution- fruits with peel color index 2-3 based on the MKADC pineapple color index guide (Figure 6) were harvested. A total of five harvest rounds (3 days interval) were conducted to clear all fruits inside the data rows. Harvested data fruits were weighed and sorted using the MKADC grading standard.

4. Fruit mass- all harvested fruits were weighed. Average fruit mass was computed using the formula:

$$\text{Fruit mass} = \frac{\sum \text{Fruit mass}}{\text{Number of data fruits}}$$

5. Plant mortality- plant mortality was gathered during the termination of the research. Missing hills and rotten plants were counted. Percent plant mortality was then computed using the formula:

$$\text{Plant mortality (\%)} = \frac{\sum \text{missing hills and rotten plants}}{\text{Number of data plants}}$$

6. Translucency rating- five samples per experimental unit per harvest round were utilized in this parameter. Fruits were cut vertically into halves. Translucency rating was determined using the hedonal rating scale as shown in Table 2. Average translucency rating was computed using the formula:

$$\text{Average translucency rating} = \frac{\sum \text{Translucency rating}}{\text{Number of fruit samples}}$$

7. Total soluble solids (TSS) - five samples per experimental unit per harvest round were utilized in this parameter. Pineapple fruit juice (10 mL) was extracted and brix was measured using an Atago handheld refractometer.

Table 2
Determination of translucency rating using the hedonal rating scale

TRANSLUCENCY RATING	DESCRIPTION	REMARKS
1	No translucence	Good fruits for export
3	Translucence affecting ≤10 % of flesh	Tolerable for export
5	Translucence affecting ≤15 % of flesh	Tolerable for local
7	Fruits with translucence affecting >15% of flesh	Reject

8. Titratable acidity (TA) - five samples per experimental unit per harvest round was utilized in this parameter. Pineapple fruit juice (10 mL) was placed inside a beaker, and 2 mL of phenolphthalein solution was added. Titration then follows by adding a basic solution (0.1 N sodium hydroxide, NaOH) to the fruit juice until the color turns to light red. The formula was then used to determine the %TA:

   TA = [volume (mL) of NaOH added x 0.1 (NaOH concentration) x 0.064 x 100] ÷ volume of juice (mL)

9. TSS/TA- five samples per experimental unit per harvest round were utilized in this parameter. This was determined using the formula:

   TSS/TA= TSS ÷ TA

Statistical Analysis

All data gathered were subjected to analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) 14 for Windows Evaluation version program. Standard deviation of means was also computed. Post hoc comparison between means was not performed since all data were not statistically different based on the ANOVA.

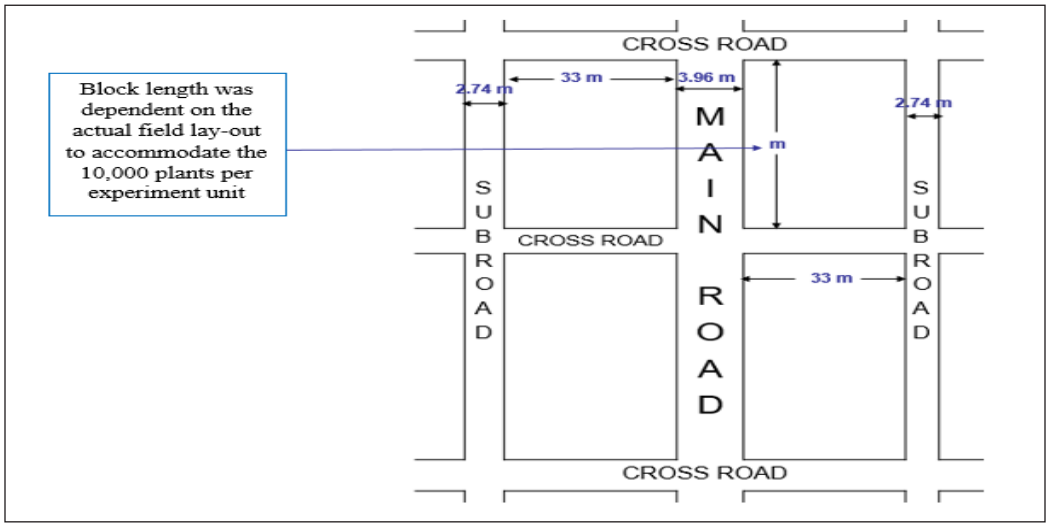

Figure 1. A typical view of the roads' dimensions which separate each experiment unit

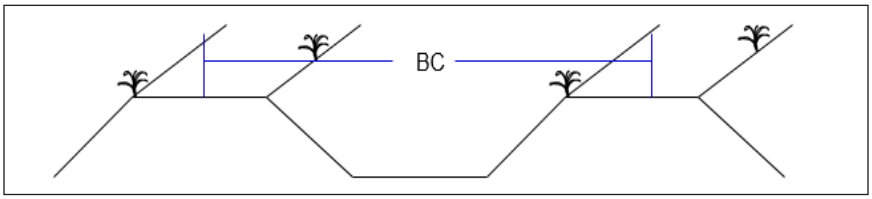

Figure 2. Typical design of a commercial pineapple seedbed (BC- distance between center of seedbeds; planting system used is quincunx)

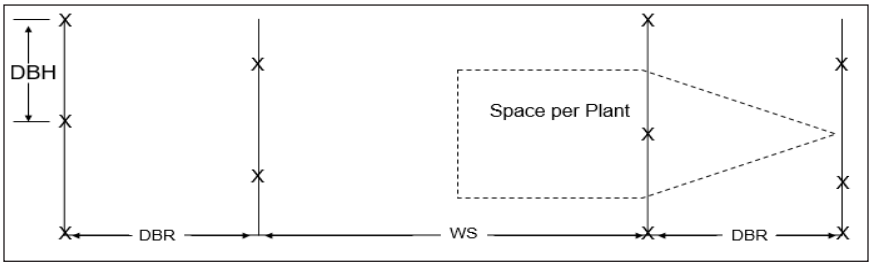

Figure 3. Top view of commercial pineapple seedbed orientation (DBH- distance between hills; DBR- distance between rows; WS- walking space)

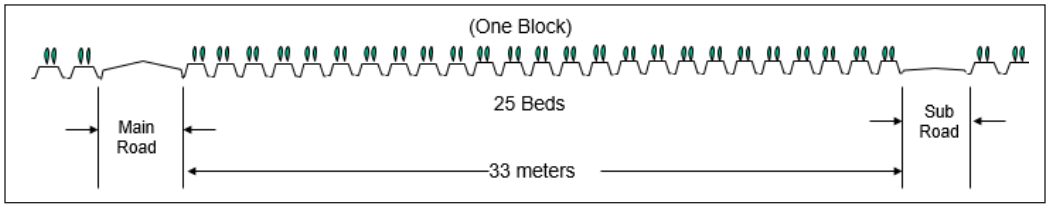

Figure 4. One pineapple block comprising 25 seedbeds with 75,000 plants per hectare serving as the control treatment

Figure 5. Photo exhibiting the height (25-30 cm) of each seedbed employed to all treatments

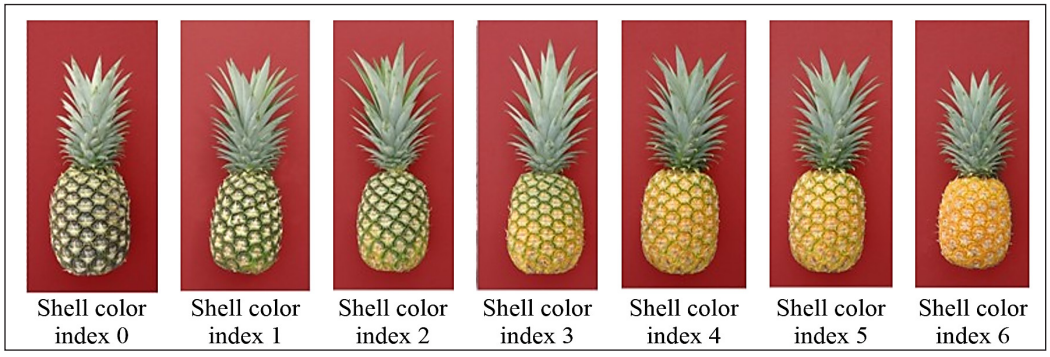

Figure 6. 'MD-2' pineapple shell color index (Source: MKADC Technical Research Group). Shell color index (SCI) 0- 0% of shell is yellow; SCI 1- 1-20% of shell is yellow; SCI 2- 21-40% of shell is yellow; SCI 3- 41-60% of shell is yellow; SCI 4- 61-80% of shell is yellow; SCI 5- 81-99% of shell is yellow; SCI 6- 100% of shell is yellow

Table 3
Dimensions of seedbed configuration per treatment

TREATMENT CODE	TREATMENT DESCRIPTION						
	Planting density hectare^{-1}	Number of rows seedbed^{-1}	Distance between hills (cm)	Distance between center of beds (cm)	Walk-space (cm)	Block width (m)	Number of seedbeds block^{-1}
T1	75000	2	19.80	134.70	83.80	33	25
T2	75000	2	21.60	120.00	69.10	33	28
T3	75000	2	24.10	110.60	59.70	33	30
T4	75000	2	25.40	105.00	54.10	33	32

RESULTS AND DISCUSSION

Plant Growth

The four seedbed configurations used in this study have comparable effects in all parameters gathered. For plant growth data, varying seedbed configurations did not influenced the plant mass (Table 4) and plant height (Table 5) of 'MD-2' pineapple plants at 6 MAP until flower induction treatment. This result implies that seedbed configurations used in the study are all feasible for 'MD-2' pineapple production. Planting density might be a better tool to improve pineapple crop growth rather than seedbed configuration.

The study of Wee (1969) revealed that pineapple leaves were longer (taller plants) and narrower at higher planting density. Moreover, Malézieux et al. (2003) reported that specific leaf mass of pineapple significantly decreased (lighter plants) at planting density higher than 6 plants m^{-2}. Although number of seedbeds block^{-1} were modified in this experiment, same planting density (75,000 planting density per hectare) was used. Hence, there were equal number of plants m^{-2} which prevented or minimized the occurrence of intraspecific competition.

Table 4
Plant mass of 'MD-2' pineapple at varying ages in response to varying seedbed configurations

TREATMENT	PLANT AGE (months after planting)			
	6	8	10	At flower induction
	Plant mass, kg			
25 seedbeds block^{-1}	0.66±0.01	1.26±0.05	1.91±0.06	2.07±0.07
28 seedbeds block^{-1}	0.71±0.03	1.24±0.08	1.74±0.04	1.80±0.05
30 seedbeds block^{-1}	0.70±0.05	1.21±0.02	1.95±0.11	2.05±0.12
32 seedbeds block^{-1}	0.65±0.04	1.17±0.08	1.77±0.13	1.84±0.12

Note: Mean ± standard deviation, values in the same column are not significantly different ($p<0.05$) by DMRT

Table 5
Plant height of 'MD-2' pineapple at varying ages in response to varying seedbed configurations

TREATMENT	PLANT AGE (months after planting)			
	6	8	10	At flower induction
	Plant height, cm			
25 seedbeds block^{-1}	63.39±1.05	74.02±1.75	91.39±1.17	98.52±1.35
28 seedbeds block^{-1}	70.37±1.58	73.45±4.20	92.76±7.30	97.50±7.51
30 seedbeds block^{-1}	68.35±2.26	72.02±2.36	97.87±1.00	102.93±2.34
32 seedbeds block^{-1}	66.33±2.16	70.70±4.39	84.05±1.79	90.93±2.26

Note: Mean ± standard deviation, values in the same column are not significantly different ($p<0.05$) by DMRT

Fruit Size Distribution and Fruit Mass

Fruit sizes distribution per hectare (Table 6) was not significantly influenced by seedbed configuration although it was noted that 25 seedbeds block^{-1} had the better fruit size distribution (with more fruits weighing above 1 kg) and higher percentage of bigger fruits. Based on author's knowledge, foreign markets such as Japan, Korea, etc. preferred fruits weighing ≥1 kg. On the other hand, the 32 seedbeds block^{-1} resulted to production of higher percentage (59.33 %) of fruits weighing below 1 kg although not significant. The results imply that modified seedbed configurations with same planting density per hectare does not affect fruit sizes distribution in 'MD-2' pineapple.

Likewise, mean fruit mass of 'MD-2' pineapple was comparable in all seedbeds block^{-1} treatments although the 28 seedbeds block^{-1} resulted to heaviest fruit mass (1.32 kg). Certainly, modifying planting density rather than seedbed configuration will result to significant differences in fruit mass of pineapple. Valleser (2018) reported that heavier fruits of 'Sensuous' pineapple was obtained in lower planting densities per hectare (45,000 to 55,000), whereas yield per hectare increases with the increasing planting density. Further, Malézieux et al. (2003) stated that yield of pineapple increased with increasing planting density per hectare. Also, Genefol et al. (2017) reported that 70,000 plants per hectare was the best planting density when compared to lower density (50,000 plants hectare^{-1}) for 'MD-2' pineapple grown in short rainy season in Southern Côte d'Ivoire. At densities above or below 74,000 plants per hectare, fruit recovery percentage as well as the quantity and quality of fruits declines (Malézieux et al., 2003).

Plant Mortality

Plant mortality was gathered during the experiment termination (last round of harvest) and still was not aggravated by the seedbed configurations used. This result means that there is a proper aeration of plants in all seedbed configurations used. Hepton (2003) mentioned that drainage and the removal of water were critical to the successful growing of pineapple, as the root system was intolerant of poorly aerated soils.

Thus, it can be concluded that plan mortality of 'MD-2' pineapple was not dependent on the various seedbed configurations as well as the spacing dimensions used in this study.

Table 6
Fruit size distribution, fruit mass and plant mortality of 'MD-2' pineapple grown at varying seedbed configurations

TREATMENT	PERCENT FRUIT SIZE DISTRIBUTION					Mean fruit mass (kg)	Percent plant mortality
	2.5-2.8 kg	2.0-2.49 kg	1.5-1.99 kg	1.0-1.49 kg	< 1.0 kg		
25 seedbeds block^{-1}	1.00	10.00	22.00	21.00	36.33	1.12±0.10	9.67
28 seedbeds block^{-1}	0.33	6.33	14.00	21.67	48.33	1.32±0.28	9.33
30 seedbeds block^{-1}	0.33	4.67	21.33	19.33	43.33	1.14±0.13	11.00
32 seedbeds block^{-1}	0.00	2.33	10.00	17.33	59.33	1.02±0.09	11.00

Note: Mean ± standard deviation, values in the same column are not significantly different ($p<0.05$) by DMRT

Physico-chemical Characteristics

In pineapple, the lower the translucency rating of fruit is the superior one. Translucency is when the pineapple flesh has a water-soaked appearance (Paull & Chen, 2003). Seedbed configurations used in this study resulted to acceptable translucency rating in pineapple compared to the set standard of MKADC farm (Castro, T. S., personal communication, November 5, 2013).

The results indicate that TSS, TA and TSS/TA of pineapple fruits were statistically comparable among treatments (Table 7). The TSS (16.80 to 17.39), TA (0.56 to 0.59) and TSS/TA (30.20 to 30.94) values surpassed the market standard which requires only a TSS value of 13, TA value of 0.5-0.7 and TSS/TA value of 20-40 ("Fresh fruit varieties", 2006). Malézieux et al. (2003) stated that at densities above or below 74,000 planting density per hectare, fruit recovery percentage as well as the quantity and quality of fruits declined. In this study however, fruit physico-chemical characteristics were similar certainly because of the constant planting density per hectare used regardless of seedbed configuration.

Table 7
Physico-chemical characteristics of 'MD-2' pineapple grown at varying seedbed configurations

TREATMENT	PHYSICO-CHEMICAL CHARACTERISTICS OF FRUITS			
	Translucency rating	Total soluble solids (TSS)	Titratable acidity (TA)	TSS/TA
25 seedbeds block^{-1}	3	16.80±0.46	0.58±0.02	30.91±2.66
28 seedbeds block^{-1}	3	17.18±0.45	0.59±0.06	30.74±4.80
30 seedbeds block^{-1}	3	16.81±0.91	0.56±0.03	30.94±0.99
32 seedbeds block^{-1}	3	17.39±0.54	0.59±0.06	30.20±2.17

Note: Mean ± standard deviation, values in the same column are not significantly different ($p<0.05$) by DMRT

CONCLUSION AND RECOMMENDATION

Results of the study revealed that the four seedbed configurations had comparable effects on the growth and physico-chemical characteristics of 'MD-2' pineapple. Hence, all seedbed configurations used in this study can be employed in the establishment of commercial 'MD-2' pineapple farm considering the dimensions of existing farm equipment. It therefore depends to end user which seedbed configuration to employ.

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A Litterbag Study: Decomposition Rate and C/N Ratio of Annual Crop Biomass Residues on An Ultisols in Natar Village, South Lampung, Indonesia

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ABSTRACT

To evaluate the decomposition rate and the C/N ratio of biomass residues from several crops that are the main food sources in Indonesia, a litterbag study was conducted from February to November 2016 on ultisols in Natar Village, South Lampung, Indonesia. There were four types of crops biomass residues used i.e., maize stovers, rice straws, shoots and leaves of cassava, and soybean stovers in the form of fresh or compost. A fifty gram dry weight of biomass was put into a litterbag, placed above the ground and at a depth of about 10-20 cm, and incubated for nine months. The remaining biomass (dry weight), the content of Organic-C, Total-N, and C/N levels of the remaining biomass in the litterbag were observed every month until nine months. The dry weight of biomass was obtained by an oven dried at 70°C for approximately 48 hours until reached the constant weight. The decomposition rates were measured as (k) = ln (X/Xo)/t. Results showed that the decomposition rate of the biomass residues of the four crops were different. The shoots and leaves of cassava biomass were most rapidly decomposed (k =-0.2830) and significantly different from others, followed by maize stover (k=-0.2066), rice straw (k = -0.1924), and soybean stover (k = -0.1675).

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Biomass of fresh or compost form and litterbag placement generally affected the decomposition rate of biomass residues.

Keywords: Biomass, carbon, crop residues, decomposition, nitrogen

INTRODUCTION

Agricultural land in Indonesia is dominated by Ultisols, which is characterized by the soil reaction (pH) is acidic, high clay content (>70 %) but low soil organic matter (SOM) (Yulnafatmawita & Adrinal, 2014). This condition brings the soil into low aggregate stability, slow infiltration and permeability rate. Therefore, the use of this soil type for agricultural land should be balanced with conservation efforts such as addition of organic matter or return of plant residues.

Crops such as rice, corn, soybeans, and cassava are seasonal crops which are widely planted as the main food source in Indonesia. The potential of biomass residues of these food crops reach 10 million tonnes per year which are usually used as animal feed or burned in piles after threshing. The potential of biomass per hectare for rice straw was up to 10 t ha^{-1}, maize and maize-soybeans inter-crops were 7,64 and 7,40 t ha^{-1} respectively (Almaz et al., 2017).

The biomass of plant wastes are easy to obtain and often abundant around farmland especially at harvest time. The plant waste/residues including the remaining biomass after the grains and other economic components are harvested. The biomass residues include the above-ground components of plant residues i.e., stems, leaves, cobs and seed shell.

Crops stover (e.g. corn stover) which is removed for producing bio fuel or animal feed gives great impact to agronomic productivity, soil and environmental quality (Blanco-Canqui & Lal, 2009; Lal, 2008; Wilhelm et al., 2007). But, there are limited information about the decomposed process of that crops residues.

Some harvesting residues are usually burned, but according to Rumpel (2008), that after 30 years, stubble burning as a regular agricultural practice did not change carbon storage or soil organic matter. Hence, return of crop residues into agricultural soils largely relates to soil biochemical and sustains organic carbon content (Chaudhary et al., 2014) and increases soil microbial biomass (Partey et al., 2014).

Utilization of plant residues as carbon (C) and nitrogen (N) reserves ready to use for soil depends on the level of decomposition and synchronization of nutrient mineralization (Murungu et al., 2011). The nitrogen availability from these residues depends on the amount of N mineralized or immobilized during decomposition. Mineralizable N is also an important indicator of the capacity of the soil to supply N for crops, in which organic materials with lower C/N ratio, N mineralization will be faster (Abera et al., 2012).

Returning plant biomass residues to the land will cause decomposition process at different rates. Decomposition was a key ecosystem process that plays major roles in determining carbon and nutrient accumulation in soils, as well as

in regulating the rate and timing of nutrient release to plant roots and soil organisms. Decomposition of organic matter is a biological process that occurs naturally and determines the amount of nutrient release (cycles) (Sariyildiz, 2008; Sayer, 2006) and the C stock in the soil (Olson et al., 2014; Singh & Gupta, 1977).

C-organic content is one of the indicators of soil fertility that affects other soil properties to support plant growth, i.e. as a source of energy and the triggers of nutrient availability for plants (Bot & Benites, 2005). Furthermore, Organic-C soil plays an important role as source and nutrient sink and as a substrate for soil microorganisms (Tornquist et al., 2009), so that the balance in soil, environment and biodiversity is maintained and sustained. Moreover, soil organic matter content affected the population and activity of soil microorganisms. There is a positive correlation between soil organic-C and soil microbial biomass carbon (Dermiyati et al., 2017).

Variations in the litter decomposition rates are mainly caused by the differences associated by litter quality, micro climates, soil properties and microbial community composition (Gholz et al., 2000; Karberg et al., 2008). Litter quality refers to characteristics of the litter (chemistry and physical attributes) that influence the susceptibility of litter to decomposition. The rates of weathering and mineralization are largely determined by the quality of the substrates in biomass, the content of nitrogen, lignin, polyphenol and the accessibility of the organic material (Brovkin et al., 2012; Silva et al., 2008). Organic materials with high lignin content and low N concentrations (high C/N ratio) have generally low decomposition rates (Johnson et al., 2007; Zhang et al., 2008). The rate of N mineralization of some legum crops tested is positively correlated with the C and N ratios, phenol and lignin levels, and their ratios with P (Cattanio et al., 2008).

Beside the litter type, environment (above or below ground environment) and site, significantly affect the decay rates of organic material (Powers et al., 2009). Environmental factors include climate and air temperature (Bothwell et al., 2014), soil temperature and soil moisture (Prescott, 2010; Zhang et al., 2008), that affect microbial activity and soil respiration (Xiao et al., 2014).

The most common and simple method to determine level of decomposition rate of residues (organic material) is a litterbag technique (Karberg et al., 2008; Kriaučiuniene et al., 2012), which allows the experimental decomposition studies under field conditions, and it can determine the rate of litter decomposition and the releases of nutrients.

Therefore, this research aimed to investigate the decomposition rate and the levels of C and N in biomass remaining after incubation of biomass residues of some crops (rice, maize, cassava, and soybeans) which were widely planted as important food crops in Indonesia.

MATERIALS AND METHODS

This research was conducted from February to November 2016 (during nine months incubation) in Natar Village, South Lampung, Indonesia (5°19'17", 105°10'28", 128 m). The soil type was Ultisol with chemical properties as follows : pH 5.17; Organic-C 1.32 %; Total-N 0.11 %; P_2O_5 (Bray-1) 12,59 mg.kg^{-1}; Potassium (K), Calcium (Ca), Magnesium (Mg), and *exchangeable* Al (cmol kg^{-1}) were 0.16, 3.23, 2.85, and 0.68, respectively.

The litterbag method was used to observe the decomposition process as natural as possible and to estimate the rate of biomass decay and its mineralization. Four types of crops biomass residue i.e. maize stover, rice straw, shoots and leaves of cassava, and soybean stover were used in the form of fresh and compost. During the decomposition process, the biomass in the litterbag was placed at two different depths which were above ground and at a depth of 10 - 20 cm. As much as 50 grams dry weight of residual biomass put into the litterbag (a nylon mesh bag with a mesh hole of about 2 mm, with the length x width of 25 cm x 20 cm), then incubated during 9 months. All treatments were repeated three times and were arranged in Randomized Complete Block Design (RCBD),. The total of litterbag incubated were 432 (4 x 2 x 2 x 3 x 9).

The litterbag started to be placed in the middle of February 2016 and the first pick up in mid March 2016, thus, every month observations were carried out on 1) The dry weight of remaining biomass, and 2) analyses the Organic-C, and Total-N of the remaining biomass. The dry weight of the biomass was obtained by oven drying at 70°C for approximately 48 hours until constant weight. The biomass weighing and analysis of Organic-C (wet digestion in $K_2Cr_2O_7$), and Total-N content (Kjeldahl method) were conducted at Laboratory of Lampung Assessment Institute of Agricultural Technology.

Decomposition Rate Calculation

The decomposition of biomass residues was monitored by the simple exponential decay model developed by (Karberg et al., 2008; Rezende et al., 1999; Silva et al., 2008), based on field and laboratory evidence of decomposition rates:

$$k = \ln (X/Xo)/t$$

$$Xo \rightarrow Xt$$

$$-d[X]/dt = k[X]$$

$$d[X_t]/[X_o] = -kdt$$

$$\int d[X_t]/[X_o] = -k\int dt$$

$$Ln\,[X_t] = -kt + Ln\,[X_o]$$

where: X= quantity of dry matter biomass after a period of time t; Xo= quantity of initial dry matter biomass; k= decomposition constant; and t = time in days. The decomposition constant (k) is generally used to compare the rate of decomposition among plant species or between different environments.

Statistic Analysis

Linear regression analysis was conducted with equation Ln $[X_t]$ = -kt + Ln $[X_o]$, to get the value of k (as a decomposition rate), followed by analysis of variance and Duncan post hoc test ($P \leq 0.01$, SAS 9,2) to indicate a significant variation within the k values of treatments. Correlation coefficients (r) between remaining biomass residues with some chemical properties and rainfall was conducted using the software SPSS Statistics version 20.0.

RESULTS AND DISCUSSION

Chemical Quality Characteristics of Biomass Residues

The content of C, N, and P of pre-incubated plant biomass was different for the four types of crops biomass residue and treatments (fresh/compost) (Table 1). The highest carbon content was found in fresh of stem soybean biomass and the lowest in shoot and leaves biomass of cassava. On the contrary for N content, the highest was found in shoot and cassava leaves in both fresh or compost biomass.

Composting reduces the level of C/N plant biomass about 20 - 30 %, the lowest C/N was in compost of biomass shoot and leaves of cassava (15.72).

Carbon and Nitrogen ratio is commonly used as a guideline for predicting the relative decomposability of organic material. The highest C/N ratio for fresh biomass is found in soybean residues, but in compost biomass, the highes was in maize biomass and the lowest being in shoot and cassava leaves biomass. Several reports showed different on the result of chemical content analysis of crops residues, this is due to differences in plant parts being analyzed. The ratio C and N of shoot of *Glycine max* was 12.7 and *Zea mays* was 49.2 (Abbasi et al., 2015). The C/N of leaves and stem of soybean and corn were 27.78, 107.34 and 30.59, 78.16, respectively (Johnson et al., 2007). Further reported that the C/N of soybean biomass residue was 47.7 - 60 (Varela et al., 2014).

Reduction of Biomass Residues During Incubation and Decomposition Rate

The biomass remaining decreased at each observation period (month) with different rates of decrease in each biomass type and treatments (fresh/compost or depth

Table 1
Initial chemical composition of biomass residues of four crops

Biomass Residue	C (%)	N (%)	C/N	P_2O_5 (%)
Maize stover-fresh	52.79	1.24	42.54	0.45
Maize stover-compost	37.18	1.09	34.01	0.44
Rice straw-fresh	48.42	1.11	43.77	0.34
Rice straw-compost	31.15	1.06	29.28	0.30
Shoot and leaves of cassava-fresh	37,78	1.94	19.44	0.54
Shoot and leaves of cassava-compost	28.46	1.81	15.72	0.52
Soybean stover-fresh	55.31	1.22	45.33	0.30
Soybean stover-compost	37.58	1.21	30.97	0.32

of incubation). In the early weeks of decomposition, the weight loss of biomass was very rapid (Figure 1). The fourth month decomposition, almost half the biomass in litterbag had been decomposed, especially for shoot and leaves of cassava which were achieved more quickly (third months). After nine months of incubation, the biomass remaining of litterbag due to decomposition was 20% (soybean biomass), 16.47% (rice straw), 12.69% (maize biomass), and 5.85% (shoot and leaves cassava). For the same external environmental conditions, the large differences in decomposition rates of the individual plant species was dependent on the initial characteristics of the biomass.

Biomass remaining of shoot and leaves cassava was lowest associated to the highest of N and P content and lowest C/N, in this case, shoot and leaves cassava contains N and P relatively higher and lowest C/N than others (Table 1). The same result had been reported by Lynch et al. (2016) that after 112 days of incubation, less than 12% of the original mass of maize residue and and only 5% of initial sorghum-sudangrass mass remained stable. It could be further explained that the difference in mass loss rate between the two residues was likely due to the lower initial C:N ratio in sorghum-sudangrass compared to maize residue. Several studies reported that after 3 to 4 months, had been decomposed half of the amount of biomass that was returned (Cattanio, et al., 2008; Lan et al., 2012). Based on these results, it is necessary to return crops biomass residues in every planting season.

Based on the regression of *natural logarithm* dry weight remaining biomass residues with time of incubation (months), the decomposition rate was obtained (slope = k) (Figure 1). Decomposition rate of biomass residues of four crops were different from one and another. Shoot and leaves of cassava biomass was the most rapidly decomposed (k = -0.2830), the next were maize stover (k = -0.2066), rice straw (average k = -0.1924), and soybean stover (k = -0.1675) respectively (Table 2).

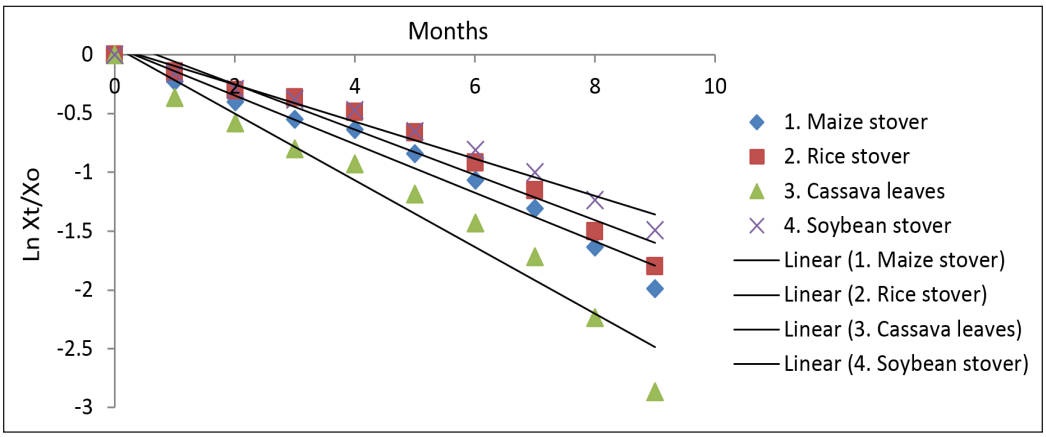

Figure 1. Regresion of *natural logarithm* dry weight of remaining biomass residues of four annual crops during nine months

Shoot and leaves of cassava biomass used in this study consisted of more leaves (about 75%) than stem and highest content of N and P, so it decomposed faster than others. After three month incubation, more than 50% shoot and leaves of cassava biomass had been decomposed (less than 50% biomass weight remaining). Whereas, soybean biomass residual used in this study consisted of more stems than leaves so that ratio of C and N was highest especially in fresh biomass. Stems showed a lower decomposition rate than the leaves of the same species, this was due to the higher C/N ratio (Lan et al., 2012). Rate of decomposition was higest for shoot and leaves of cassava biomass due to the high quality of this organic material, which had a relatively high N and P content, and had lowest C/N ratio.

The similar results had also been reported by Johnson et al. (2007) that decomposition of crop residues was strongly influenced by residues material quality that varied among species and organs within species.

Table 2
Regression equations and decomposition rate (k) of biomass residues of four annual crops

Crops Residues	Regression equations	R^2	k
Maize stover	y=-0.2066x + 0.0637	0.9713	-0.2066
Rice straw	y=-0.1924x + 0.1305	0.9535	-0.1924
Shoot and leaves of cassava	y=-0.283x + 0.0627	0.9554	-0.2830
Soybean stover	y=-0.1675x + 0.0594	0.9758	-0.1675

Decomposition rate of soybean stover biomass was lowest, related to the dominant part of the plant biomass used in this study which was the stem and only a few leaves. This was due to differences in the initial chemical composition of the crop residues, the most important is indicated by the C:N (Zhang et al., 2008). Organic matter decomposition rates are influenced by the quality of residues depending on the plant species. The initial quality of organic matter of cassava shoot and leaves biomass was better than others, indicated by higher initial N and P content. Lignin and N contents (C/N) of the plant residues play an important role in their decomposition, related to microbial cell synthesis requiring nitrogen. However, lower availability of nitrogen temporarily reduces soil microbial activity, and certainly affects the decomposition of plant residues.

The litter quality is the most important direct regulator of litter decomposition at the global scale. The data synthesis revealed that there was significant relationship between litter decomposition rates and the combination of climatic factor and litter quality (Zhang et al., 2008). Further, reported that plant residue decomposition under specific conditions of temperature and moisture was a function of a plant chemical and biochemical quality (Nourbakhsh, 2006). Decomposition rate was affected by both litter quality and stream. However,

the relative importance of litter quality decreased through time, explaining 97% of the variation in the first week but only 45% by week 8 (LeRoy & Marks, 2006).

Effect of Type Plant Biomass, Fresh/ Compost and Litterbag Placement

Based on analysis of variance, the main effect (four type plant biomass residues, biomass form (fresh/compost), and depth of application) on decomposition rate (k) were significantly different (Table 3). Further, the analysis of these three factors interaction (Biomass*Fresh/Compost*Depth) was also significantly different.

Table 3
*Analysis of variance of decomposition rate values (k) of treatments (significantly *P < 0.05, **P < 0.01,n=3)*

Source	DF	F Value	Pr > F
Replication	2	4.09	0.0268
Biomass	3	186.35	<.0001
Fresh/Comp	1	57.75	<.0001
Depth	1	41.83	<.0001
Biomass* Fresh/Comp	3	1.82	0.1641
Biomass*Depth	3	2.69	0.0641
Fresh/Comp *Depth	1	1.86	0.1827
Biomass*Fresh/Comp *Depth	3	5.44	0.0041

Based on Figure 2, compost biomass was decomposed faster than fresh biomass, and biomass placement above or below

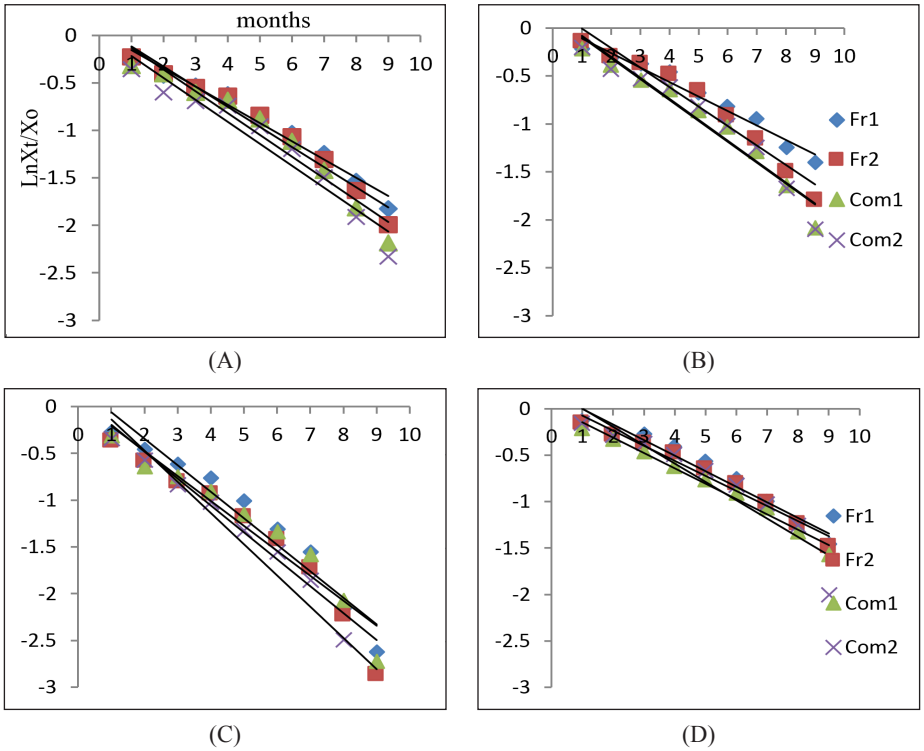

Figure 2. Regression of Ln Xt/Xo and time (months) of biomass residues of four crops (A = maize stover; B = Rice straw; C = Cassava leaves; D = Soybean stover), the treatments : fresh and above ground placed (Fr1), fresh and 10-20 cm depth (Fr2), compost and above ground placed (Com1), compost and 10-20 cm depth (Com2)

ground (10 – 20 cm) overall affected decomposition rate. Decomposition rate of fresh biomass of maize stover placed at a depth of 10 - 20 cm was significantly faster than above ground (Table 4), as well as of fresh biomass of rice straw and compost of soybean stover placed at a depth of 10 - 20 cm decomposed significantly faster than above ground. Compared to all treatments, shoot and leaves of cassava compost was the fastest decomposed biomass when placed at 10 – 20 cm depth (k = -0.3336). Litter type, decomposition environment, and site significantly affect decay rates (Powers et al., 2009).

Table 4
Decomposition rate (k) and correlation (r) of fresh and compost biomass residues of four annual crops during nine months incubation

Biomass Residue	Regression Equations	r	k
Maize stover			
Fresh, above ground	y=-0.1925x + 0.0403	-0.996**	-0.1925 c*
Fresh, 10 – 20 cm depth	y=-0.2119x + 0.0982	-0.996**	-0.2119 b
Compost, above ground	y=-0.2296x + 0.1021	-0.998**	-0.2296 b
Compost, 10 – 20 cm depth	y=-0.2312x + 0.0147	-0.992**	-0.2312 b
Rice Straw			
Fresh, above ground	y=-0.1507x + 0.0394	-0.996**	-0.1507 d
Fresh, 10 – 20 cm depth	y=-0.2033x + 0.1989	-0.997**	-0.2033 bc
Compost, above ground	y=-0.2184x + 0.1257	-0.997**	-0.2184 b
Compost, 10 – 20 cm depth	y=-0.2187x + 0.1385	-0.994**	-0.2187 b
Shoot and leaves of cassava			
Fresh, above ground	y=-0.2728x + 0.2197	-0.995**	-0.2728 a
Fresh, 10 – 20 cm depth	y=-0.2879x + 0.094	-0.991**	-0.2879 a
Compost, above ground	y=-0.2865x + 0.0558	-0.984**	-0.2865 a
Compost, 10 – 20 cm depth	y=-0.3336x + 0.195	-0.983**	-0.3336 a
Soybean stover			
Fresh, above ground	y1=-0.1678x + 0.167	-0.996**	-0.1678 d
Fresh, 10 – 20 cm depth	y=-0.1625x + 0.0908	-0.999**	-0.1625 d
Compost, above ground	y=-0.1656x + 0.0209	-0.997**	-0.1656 d
Compost, 10 – 20 cm depth	y=-0.1982x + 0.2046	-0.996**	-0.1982 c

*Mean values followed by same letter do not significantly different ($P \leq 0.01$), mean of three replicates

Theoretically, greater contact with the microbial community in buried residues was responsible for higher rates of decay in comparison to surface-placed residue. Further, it was reported that generally carbon losses through microbial respiration were significantly higher when litters were incubated with soils, than without soils, probably due to differences in biotic and abiotic factors (Silveira et al., 2011). Surface or buried-placed affect C and N mineralization, higher moisture content and retention is often associated with increased rates of decomposition in buried

than surface-placed residues in laboratory incubation and litterbag field studies (Lynch et al., 2016).

Carbon Decomposition, Nitrogen Remaining and C/N

Carbon/Nitrogen ratio is commonly used as a guideline for predicting the relative decomposability or N mineralization potential of organic materials added to soil (Reddy et al., 1979). The decomposition process of biomass lowers carbon content. In this study, total C in biomass remaining after nine months incubated at litterbag study (in averages) were 13.84% for maize stover, and 13.79%, 9.98%, 16.64% respectively for rice straw, shoot and leaves of cassava, and soybean stover biomass (Table 5). C and N in compost biomass was faster mineralized than fresh biomass, this associated to high C/N ratio (low N) of the fresh biomass resulting in immobilization of inorganic N in microorganism. It can be further reported that organic material decomposition was negatively related to soil C/N ratio (R^2=0.66; P<0.001, the relationship was determined by two monoculture maize plots that both had a relatively high C/N ratio and slow SOM decomposition (Cong et al., 2015).

Table 5
Total C in remaining biomass at litterbag observed in nine months decomposition of biomass residues of four annual crops

Treatments	C (%)								
	1	2	3	4	5	6	7	8	9
A. Maize stover									
Fresh,above ground	46.64	35.82	32.06	30.74	28.58	25.59	23.08	19.25	16.59
Fresh,10–20cm	44.74	35.90	30.88	28.74	26.94	23.75	20.77	17.74	15.52
Compost,above	35.60	29.88	27.09	25.30	21.53	18.39	16.34	14.63	12.25
Compost, 10 – 20 cm	34.29	31.50	26.21	24.88	20.79	15.08	13.86	12.09	10.75
B. Rice straw									
Fresh,above ground	42.73	35.43	29.82	27.54	24.20	21.93	19.39	17.99	16.84
Fresh,10–20cm	41.48	36.78	28.58	25.20	22.82	21.73	19.24	16.53	15.36
Compost,above	32.22	29.06	21.62	21.38	20.00	17.77	15.77	14.77	11.66
Compost, 10 – 20 cm	30.00	28.01	21.05	20.96	19.43	16.98	14.89	13.67	11.30
C. Cassava Shoot									
Fresh,above ground	33.15	30.89	23.83	20.58	17.80	15.79	13.61	12.08	10.25
Fresh,10–20cm	31.65	30.06	22.09	20.14	17.42	15.34	12.97	12.16	10.53
Compost,above	28.00	26.41	18.61	17.77	16.03	15.36	13.04	11.38	9.74
Compost, 10 – 20 cm	27.34	25.88	18.58	16.96	15.29	1529	12.57	10.71	9.43
D. Soybean stover									
Fresh,above ground	46.49	41.50	33.82	31.20	28.53	25.37	23.04	20.12	19.17
Fresh,10–20cm	44.17	42.12	33.20	30.19	26.56	24.21	22.45	19.78	18.73
Compost,above	37.31	35.27	28.82	26.32	22.08	20.03	17.77	15.90	13.65
Compost, 10 – 20 cm	37.88	34.45	26.74	24.66	20.21	18.58	16.81	14.38	12.61

The decomposition of crop residues is the result of complex microbial processes controlled by numerous factors. Based on multiple regression analysis, combination of C:N ratio and lignin concentration in crop residues explains decomposition rates of 73% for the controlled variation (Kriaučiuniene et al., 2012). This indicates that this parameter could be used to predict decomposition rates. Decomposition rate is correlated with the amount of microorganism, there was a significant positive effect of internal N concentration on litter decomposition, mainly because the N requirement of the decomposers.

Carbon losses through microbial respiration were significantly higher when litters were incubated with soils, probably due to differences in biotic and abiotic factors (Silveira et al., 2011). A linear relationship between net N mineralization and CO_2 evolution was reported by Gilmour et al. (1985) for sewage sludge and four plant materials having a high total N content. A highly correlated linear relationship existed between N mineralization and CO_2 evolution during the study for digested biomass sludges but not for the fresh plant biomass.

During the decomposition carbon was used as an energy source by decomposers while nitrogen was assimilated into cell proteins and other compounds. Thus, with increasing decomposition time, the levels of C would decrease and nutrient released from the plant residues (especially N). The N mineralization pattern of the residues closely reflected the differences in their chemical composition (Chaudhary et al., 2014).

C/N of rice straw fresh placed at 10 – 20 cm depth decrease from 37.71% to 18.89% after nine months, as well as other biomass residues (Table 6). Decrease in C/N and a higher nitrogen content in the original biomass material promoted decomposition faster. A combination of N, C:N ratio and lignin concentration in crop residues explained decomposition rates of more than 70%, and the rest of it depending on the other factors such as plant type, part and environment (Kriaučiuniene et al., 2012).

This result agrees with some studies that litter with high N contents (low C/N ratios) decomposed significantly faster than litter with low N contents (Kara et al., 2014; Tripathi et al., 2006). Further, a study of leaf

Table 6
C/N in remaining biomass at litterbag observed in nine months decomposition of biomass residues of four annual crops

Treatments	C/N (%)								
	1	2	3	4	5	6	7	8	9
A. Maize stover									
Fresh,above ground	37.61	34.01	32.51	31.39	29.98	28.76	26.32	22.13	19.14
Fresh,10–20cm	36.70	33.50	31.76	28.18	28.47	26.99	23.88	20.47	18.11
Compost,above	31.62	28.63	26.91	26.84	24.37	21.79	19.60	17.83	15.01
Compost, 10 – 20 cm	29.34	27.74	25.72	25.33	22.93	18.54	17.25	15.50	13.90

Table 6 *(continue)*

Treatments	C/N (%)								
	1	2	3	4	5	6	7	8	9
B. Rice straw									
Fresh,above ground	41.49	35.91	31.39	30.05	28.14	25.90	24.34	22.76	21.50
Fresh,10–20cm	37.71	35.72	30.61	29.17	27.28	25.99	23.37	20.09	18.89
Compost,above	30.69	27.67	25.81	25.34	24.09	21.67	19.87	18.45	14.82
Compost, 10 – 20 cm	27.78	26.26	24.47	23.60	23.22	20.53	18.61	17.09	14.37
C. Cassava Shoot									
Fresh,above ground	17.54	17.45	17.04	16.73	14.72	13.57	11.97	10.92	9.02
Fresh,10–20cm	17.08	17.08	16.57	15.91	14.28	13.08	11.21	10.93	9.21
Compost,above	16.05	15.85	15.05	14.95	13.55	13.03	11.24	9.95	8.45
Compost, 10 – 20 cm	15.71	14.90	15.33	14.62	13.30	13.30	11.00	9.54	8.33
D. Soybean stover									
Fresh,above ground	38.64	36.52	33.51	30.30	29.83	26.89	24.43	21.49	20.69
Fresh,10–20cm	37.23	35.61	32.66	30.50	28.25	25.48	23.63	20.90	19.78
Compost,above	30.11	28.06	27.88	27.93	24.17	22.09	19.60	17.61	15.05
Compost, 10 – 20 cm	29.08	27.10	27.93	25.86	21.67	20.49	18.68	16.28	14.23

litter reported that C/N ratio to be a better predictor of mass loss than the lignin/N ratio in a microcosm decomposition (Taylor et al., 1989).

Based on results of correlation test, remaining biomass (%)−significantly correlated with C, N, and C/N on the sixteen treatments (Table 7). The highest correlation was with the C/N. Average correlation coefficients (r) of remaining biomass (%) with C, N, and C/N were 0.948, 0.807, and 0.971 respectively.

To accelerate the decomposition of plant material whose high C/N ratio was composted first, as was often done. The same result was reported, that mixing maize

Table 7
Pearson correlation coeficients (r) between remaining biomass residues (%) and some chemical properties (C, N, and C/N) after nine months incubation (n = 3)

Treatments	C (%)	N (%)	C/N
Maize stover, fresh, above ground	0.96**	0.84**	0.97**
Maize stover, fresh, 10-20 cm depth	0.97**	0.88**	0.98**
Maize stover, compost, above ground	0.98**	0.90**	0.99**
Maize stover, compost, 10-20 cm depth	0.96**	0.92**	0.99**
Rice straw, fresh, above ground	0.91**	0.97**	0.96**
Rice straw, fresh, 10-20 cm depth	0.90**	0.76*	0.98**
Rice straw, compost, above ground	0.96**	0.73*	0.99**
Rice straw, compost, 10-20 cm depth	0.95**	0.76*	0.99**
Shoot and leaves of cassava, fresh, above ground	0.97**	0.80**	0.98**
Shoot and leaves of cassava, fresh, 10-20 cm depth	0.97**	0.83**	0.95**

Table 7 *(continue)*

Treatments	C (%)	N (%)	C/N
Shoot and leaves of cassava, compost, above ground	0.93**	0.67*	0.96**
Shoot and leaves of cassava, compost, 10-20 cm depth	0.97**	0.74*	0.95**
Soybean stover, fresh, above ground	0.92**	0.75*	0.98**
Soybean stover, fresh, 10-20 cm depth	0.94**	0.95**	0.99**
Soybean stover, compost, above ground	0.99**	0.76*	0.99**
Soybean stover, compost, 10-20 cm	0.92**	0.63*	0.99**
Avarage	**0.95**	**0.81**	**0.97**

residues with other plant residues whose C/N was lower (*V. Faba* or *T. Diversifolia*) could increase N mineralization and decrease C/N (Partey et al., 2014).

The Effect of Climate Factors on Decomposition Rate of Biomass Residues

Climate factors such as temperature and soil moisture (rainfall) affect litter decomposition. In this study, the first three months incubation, rainfall was high (> 300 mm every month) (Table 8), thus supporting the decomposition intensively.

Based on analyses of variance, rainfall significantly affects on biomass remaining (%) ($p<0,05$) and significant correlated (pearson correlation = 0.783) (Table 9). Rainfall affects soil moisture, according to a research report, monthly rainfall and soil moisture varied over sampling period but in similar patterns in both land uses observed

Table 8
Avarage remaining biomass (%) and rainfall (mm) during nine months incubation

	Month								
	1	2	3	4	5	6	7	8	9
Avarage remaining biomass (%)	78.82	66.35	58.86	52.67	43.00	35.21	28.05	19.86	13.75
Rainfall (mm)*)	330.8	305.7	332.7	195.7	185.1	118.5	107.9	54.4	103.2

*)Data was processed from the Meteorological Station at Branti village which is about 10 km from this research location

Table 9
Correlation coeficients (r) between avarage remaining biomass and rainfall after nine months incubation

Correlation			
		Rainfall	Remaining Biomass
Rainfall	Pearson Correlation	1	.783*
	Sig. (2-tailed)		.013
	N	9	9
Biomass (%)	Pearson Correlation	.783*	1
	Sig. (2-tailed)	.013	
	N	9	9

* Correlation is significant at the 0.05 level (2-tailed)

(Abera et al., 2012). Soil moisture was most important during the early decomposition stage rather than the late stages, but under field experimental conditions, however, it is difficult to detect the net effects of moisture on respiration or decomposition because all factors interact to affect the litter decomposition (Virzo De Santo et al., 1993).

Furthermore, litter decomposition increased with increasing temperature in the high moisture and decreased with increasing temperature in the low moisture (Butenschoen et al., 2011). The sensitivity of soil respiration and litter decomposition to soil temperature is influenced not only by soil water but also by various factors including soil nutrition and litter quality. Berrier et al. (2014) reported that both plant litter type and incubation site were important in determining decomposition rates and that when all litter types were considered, the mean decomposition rate for plant material placed in the wetland was significantly higher than for the upland site ($k_{wetland} = 0.42 \pm 0.02$, $k_{upland} = 0.29 \pm 0.02$).

CONCLUSION

Decomposition rate of residues biomass of the four crops was different from each other. After nine months of incubation, the biomass remaining and decomposition rate (k) of shoot and leaves cassava were the most rapidly decomposed (5.85%, k = -0.2830), followed by maize stover (12.69%, k=-0.2066), rice straw (16.47%, k = -0.1924), and soybean stover (20%, k = -0.1675), this due to initial characteristics (levels of C, N, and P) and C/N.

Decomposition of compost biomass was significantly faster than fresh biomass for maize stover and rice straw. Litterbag placement above or below ground (10 – 20 cm) overall affected decomposition rate. Fresh biomass of rice straw and shoot and leaves of cassava in litterbag placed at a depth of 10 - 20 cm decomposed significantly faster than above ground.

Remaining weight of biomass (%) most correlated with C/N compared to C or N content (%) after nine months incubation (the r value respectively 0.97; 0.95, and 0.81). Monthly rainfall has significant effect on decomposition rate (r = 0.783), where in the first months (with high rainfall each month) more intensive decomposition than in the following months.

This research will be continued with the application of the biomass residues in the field and will observe the microbial activity of the soil.

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A Preliminary Study on the Diversity and Abundance of *Onthophagus* Species (Coleoptera: Scarabaeidae) in an Oil Palm Plantation, Peninsular Malaysia in Relation to Carbon Dioxide and Soil Organic Matter

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ABSTRACT

Dung beetles are important bioindicator species in an ecosystem. The relationship between the CO_2 concentration and percentage of soil organic matter (SOM) with the diversity and abundance of *Onthophagus* species was investigated as a model genus in a model sampling area in an oil palm plantation in the southern part of Peninsular Malaysia. In total, 554 samples belonging to 25 species of *Onthophagus* were collected. No significant differences in CO_2 concentration during the day and night across the stations were found, except DCO_2S1 vs DCO_2S2 ($p = 0.014$, $p < 0.05$). Also, the diversity and abundance of species did not differ significantly (H'= 2.250 [diurnal] and H'= 1.854 [nocturnal]; $p > 0.05$). The dung beetle species recorded specifically during the day were *O. aphodiodes*, *O. denticollis*, *O.* cf. *pacificus*, *O. dayacus*, *O.* sp. 1, *O. penicillatus*, *O. peninsulocupreus*, and *O.*"hairy group" (H'= 1.739), while *O. babirussoides*, *O. phaenids*, *O. insicus*, *O. paraphamaeomorphos*, *O. peninsularis*, and *O. parachandrai* (H'= 1.677) were observed at night. Furthermore, no relationship was discerned between % SOM vs diversity indices (r = 0.348), evenness (r = -0.289), and richness (r = 0.972) of dung beetles. This is the first study in Malaysia to illustrate a lack of relationship between CO_2 concentration and SOM with *Onthophagus* spp. The preliminary data of this research

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can be used for future studies on diversity and ecology of dung beetles in relation to CO_2 and SOM.

Keywords: CO_2, correlation, diurnal, Malaysia, nocturnal, *Onthophagu*s, Scarabaeidae, SOM

INTRODUCTION

The family Scarabaeidae, which includes dung beetles is one of the largest families of beetles, with around 30,000 recorded species worldwide (Hanski & Cambefort, 1991). According to Hernandez (2001), dung beetle species can be classified as nocturnal or diurnal, depending on when they are active. Species that are active after sunrise and before sundown are considered diurnal, whereas those that are active only at nighttime are considered nocturnal. Based on Ellen (2002), more species and abundance of dung beetles with larger mean size were captured per trap during nocturnal trapping periods compared to diurnal trapping periods.

According to a study by Montes and Halffter (1995), *Onthophagus landolti*, which colonizes both horse and cow dung, is normally active between 10:00 and 11:30 h (pers. obs. E. Montes De Oca). However, colonization of dung by dung beetle species is more common at nighttime than daytime. Diurnal colonization activity is also subject to seasonal variations. Due to the exploitation of dung by crepuscular and nocturnal species, diurnal dung beetle species are less numerous than nocturnal species. In general term, diurnal colonization activity is lower than nocturnal colonization activity.

In the tropics, soil organic matter (SOM) determines the productivity and fertility of the soil, especially in highly weathered soil, where the nutrient reserves are limited or none, and the soil is managed without any external inputs of inorganic and organic fertilizers (Ywih et al., 2009). The conversion of biomass into humus (including humic acids), translocation and aggregation of carbon into subsoil prevent carbon oxidation and lead to soil organic carbon (SOC) sequestration. By moving and breaking down excrement and incorporating organic matter in the soil, the activities of dung beetles influence the availability of organic matter (Shang & Tiessen, 1997), which indirectly increase the level of soil fertility (Avendano-Mendoza et al., 2005). Dung beetles also carry out bioturbation processes (Nichols et al*.,* 2008) and the recycling of the nutrients (Estrada & Coates-Estrada*,* 1991).

Dung burial activity by dung beetles has positive effects on soil fertility (Bang et al*.,* 2005). Dung decomposition involves a range of organisms and is primarily an abiotic process. Dung pads left on fields release greenhouse gases, including CO_2 and methane. The decomposition process of dung pads by dung beetle releases greenhouse gas (GHG) fluxes, which are considered one of the abiotic factors, similar as temperature and moisture or plant and soil interactions, whereas the role of the dung fauna has received far less attention (Penttila et al., 2013).

Hutton and Giller (2003) had reported greater beetle biomass, diversity, and species richness in organic farms compared to rough and intensive grazing sites, located in County Cork and County Tipperary in Southern Ireland. They also found that the abundance of some dung beetle species, such as *Aphodius*, was significantly lower in spring and autumn on the rough and intensive farms compared to the organic ones. The biomass of *Aphodius*, however, might be considerably higher in spring, late summer, and autumn for natural and rough grazing agriculture compared to intensive agriculture. Using the detrended correspondence analysis of dung beetle assemblages from the variously managed farms, Hutton and Giller (2003) found that the seasonal abundance of dung beetle species was greater on the organic farms than the rough and intensive farm.

The objective is to study both CO_2 concentration during day and night (air parameter) and percentage of soil organic matter (SOM) (soil parameter) in the oil palm plantation and its association towards the diversity and abundance of diurnal and nocturnal *Onthophagus* spp. dung beetles.

MATERIALS AND METHODS

Study Area

This research was carried out at three different stations in an oil palm plantation areas located at Felda Lui Muda, Negeri Sembilan in the southern part of Peninsular Malaysia. The stations were separated apart and located at the coordinates N 03° 03' 15.1", E 102° 22' 02.7 (station 1), N 34° 00' 00.0", E 068° 54' 22.5 (station 2) and N 63° 03' 45.0", E 068° 54' 22.5 (station 3). The coordinates were recorded using GARMIN GPS 12 XL (12 channel).

Sampling Activity of the Dung Beetles

The adults of *Onthophagus* spp. were collected using baited pitfall traps from October 2015 until June 2016 by conducted three sampling activities for each station. Fifteen traps were placed randomly in each station, at a minimal distance of 5 m from each other. The diameter of the pitfall (bucket) traps was 17.5 cm, the circumference was 63 cm, and the height was 15.5 cm. Due to easy availability, the rotten fish was chosen as the bait. It was filled into a small cup and placed at the center of each trap. The baited pitfall traps were filled with soapy water (one-quarter of the volume of the trap) to ensure that no samples escaped from the trap. The traps were inspected four times in every 24-h cycle (12 hours interval on separate days and nights) for every sampling activity. The dung pads was also observed in each station during sampling activities. The dung beetles were collected in these two periods to identify the behavior and diel flight activities of the species: after sunrise (nocturnal) and after dark (diurnal). The adult sample of dung beetles were preserved in 70% alcohol.

Soil Sampling and Analysis of SOM

Soil samples were collected from all the stations, with three replications for each station. The samples of the soil were randomly collected from three holes that

had been dug (at a depth of 15.5 cm depth) to fit the baited pitfall traps. The total of the soils collected were nine samples. The samples were wrapped in an aluminum wrapper and placed in a box to maintain the soil composition. These samples were used to determine the percentage of SOM. The soil analysis was done in accordance with Ministry of Agriculture, Fisheries and Food (MAFF) (1982) guidelines within a week after sampled.

Measurement of CO_2

The concentration of CO_2 was measured using a CO_2 meter (version 8802-EN-00) in ppm units. The CO_2 levels were recorded during sampling activities at each site during the day (06.30 – 07.00 am) and night (19.00 – 19.30 pm) with three replications for each site. CO_2 measurements were collected at that particular time because it was an active time for the diurnal (from six in the morning) and after 18-hour for nocturnal species of dung beetles (de Oca T. & Halffter, 1995). The total of nine CO_2 concentration data was recorded at both daytime and nighttime per sampling site.

Identification and Recording of the Dung Beetle Samples

The adult dung beetle specimens were identified to the species level using a stereomicroscope based on the pictorial guide (Ek-Amnuay, 2008) and dry specimens from the Center of Insect Systematics (CIS), Universiti Kebangsaan Malaysia collections.

Statistical Analysis of Species Diversity and Richness

Species diversity and abundance were analyzed using Paleontological statistics software package for education and data analysis (PAST), while Minitab 17 was used to run a *t*-test and One-Way ANOVA ($p < 0.05$) (Minitab, 2016). Microsoft Excel was used to create the figure of CO_2 level, and PC ORD was used to run a multivariate statistical analysis of ecological communities.

RESULTS

Species Diversity and Abundance of Dung Beetles

The total of 554 adult beetles was obtained from the three sampling localities. They were identified to belong to 21 species with four species not definitely identified and were temporarily designated as *Onthophagus* sp. 1, sp. 2, sp. 3 and "hairy group" (Table 1). Diurnal species (289 individuals belonging to 19 species) consist more individuals than nocturnal species (265 individuals belonging to 17 species). The *Onthophagus* spp. found in the daytime accounted for 52.17% of the total number, while the other 47.83% of individuals were recorded at night. Of the 25 species recorded, 11 were recorded both during the day and night, eight during the day, and six at night.

With regard to diurnal species, the dominant species was *O. crassicolis* (n = 69), followed by *O. pedator* (n = 68), and *O. rugicollis* (n = 45). *Onthophagus pedator* (n = 106) was the most abundant nocturnal species, followed by *O. rugicollis* (n =

Table 1
Diversity and abundance of the nocturnal and diurnal Onthophagus *spp. in Felda Lui Muda, Serting, Malaysia*

No.		Diurnal	Nocturnal
1	*Onthophagus pacificus*	13	30
2	*Onthophagus crassicolis*	69	25
3	*Onthophagus pedator*	68	106
4	*Onthophagus waterstradti*	13	14
5	*Onthophagus rugicollis*	45	56
6	*Onthophagus orientalis*	4	1
7	*Onthophagus rutilans*	6	3
8	*Onthophagus cervus*	16	12
9	*Onthophagus proletarius*	8	4
10	*Onthophagus* sp. 2*	2	3
11	*Onthophagus* sp. 3*	16	2
12	*Onthophagus parachandrai*	0	1
13	*Onthophagus insicus*	0	2
14	*Onthophagus paraphamaeomorphos*	0	1
15	*Onthophagus peninsularis*	0	3
16	*Onthophagus babirussoides*	0	1
17	*Onthophagus phaenids*	0	1
18	*Onthophagus aphodiodes*	9	0
19	*Onthophagus denticollis*	1	0
20	*Onthophagus "*hairy group"*	1	0
21	*Onthophagus* cf. *pacificus*	1	0
22	*Onthophagus dayacus*	2	0
23	*Onthophagus* sp. 1*	8	0
24	*Onthophagus penicillatus*	5	0
25	*Onthophagus peninsulocupreus*	2	0
TOTAL		289	265

*Temporarily designated

56), and *O. pacificus* (n = 30). Only one individual of *O.* cf. *pacificus*, *O.* hairy group, and *O. denticollis* were found specifically during the day, whereas *O. babirussoides*, *O. phaenids*, *O. paraphamaeomorphos*, and *O. parachandrai* were found only at night. The other species were active both during day and night have been highlighted in Table 1.

Determination of *Onthophagus* sp. diversity during the day and night showed through the analysis of Shannon-Weiner (H'). Shannon's index accounts for both abundance and evenness of the species present, which consider proportions and not an absolute number (Gamito, 2010). Based on the data collected, the diurnal and nocturnal diversity index (H') were 2.250 and 1.854, respectively. The Shannon (H') diversity index of the dung beetles at Stations 1, 2, and 3 were 2.056, 1.974, and 2.045, respectively. Even though there are different types of species specifically

found at daytime and night time, but there was no significant difference in nocturnal and diurnal species abundance (p = 0.074). The diurnal species recorded during daytime only were *O. aphodiodes, O. denticollis, Onthophagus* "hairy group"*, O. pacificus, O. dayacus, Onthophagus* sp. 1*, O. penicillatus and O. peninsulocupreus* (H') = 1.739, and specifically found at night were *O. parachandrai, O insicus, O. paraphamaeomorphos, O. peninsularis, O. babirussoides* and *O. phaenids* (H') = 1.677.

A high diversity index denotes high abundance and rare species. Although higher numbers of dung beetles were recorded at Station 2(n = 319) and Station 3 (n = 179) than Station 1 (n = 56), Station 1 consisted of more species with equal abundance than the other two stations. Diurnal diversity index is higher than nocturnal because as influenced by the abundance of *Onthophagus* sp. 1, *O. aphodiodes* and *O. penicillatus* (Table 1). There was no significant difference between the diurnal and nocturnal abundance of *Onthophagus* spp., with p = 0.877 (p > 0.05), while the abundance between stations; S1 vs S2 (p = 0.053), S1 vs S3 (p = 0.108) and S2 vs S3 (p = 0.345), also showed non-significant difference between stations.

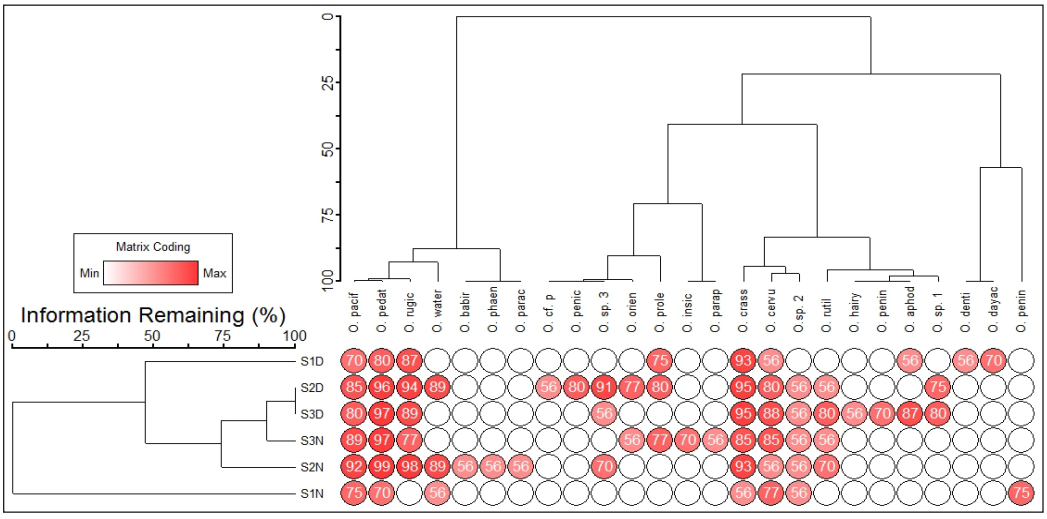

Figure 1. Abundance of diurnal and nocturnal *Onthophagus* spp. from three stations at Felda Lui Muda, Serting
S = station; D = diurnal; N = nocturnal

At 75% of similarity, the samples were divided into three different groups. The first group was S1D, the second group including S2D, S3D, S2N, and S3N, and the third group was S1N. Based on Figure 1, the highest presence probability of dung beetle found was *O. pedator*, while, the lowest abundance of dung beetle were *O. babirussoides, O. phaenids, O. parachandrai*, *O.* cf. *pacificus*, *O. paraphamaeomorphos, Onthophagus* "hairy group" and *O. denticollis*. S2D and S3D from group 2 showed very close

relationship between the diversity and presence probability of *Onthophagus* spp. The diversity and presence probability of *Onthophagus* spp. was the lowest at Station 1 in group 3 during the night (S1N). Group 3 was considered as the outgroup because the diversity and presence probability of *Onthophagus* spp. shown do not match with other groups.

Day and Night CO_2 Concentration

As shown in Table 2, based on one-way ANOVA analysis of the day and night CO_2 concentration readings from three different stations, there was a significant difference in CO_2 concentrations between DCO_2S1 vs DCO_2S2 ($p = 0.014$, $p < 0.05$) only. Overall, based on two-sample *t*-test, no significant difference was shown between air CO_2 concentration of day and night time with *p*-value = 0.303 ($p > 0.05$).

Table 2
One-way ANOVA of CO_2 concentration between three stations

Station	DCO_2S1	DCO_2S2	DCO_2S3	NCO_2S1	NCO_2S2
DCO_2S2	**0.014**				
DCO_2S3	0.489	0.622			
NCO_2S1	0.868	0.390	0.996		
NCO_2S2	0.926	0.397	0.993	1.000	
NCO_2S3	1.000	0.101	0.784	0.968	0.984

D= day; N= night; CO_2=carbon dioxide; S=station; Value in bold show significant difference ($p < 0.05$)

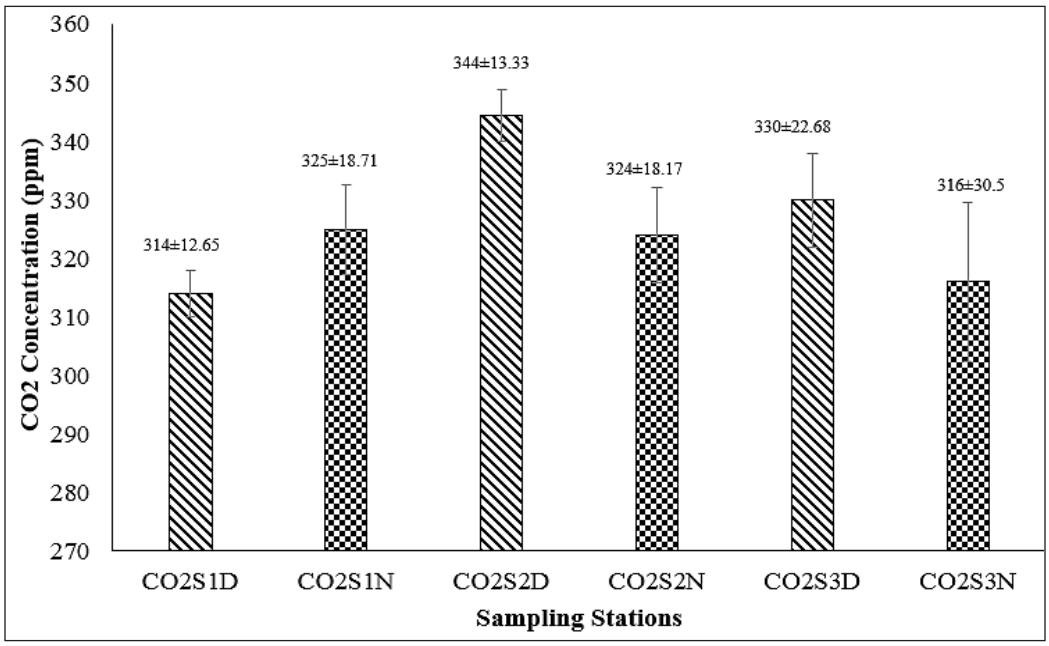

Figure 2. CO_2 concentration during day and night time at station 1, station 2 and station 3

Generally, the CO_2 concentration was higher during the daytime than at nighttime. The highest CO_2 concentrations were recorded at Station 2 during daytime, with 344 ppm (daytime), while at night, Station 1 shows the highest CO_2 concentration with 325 ppm (Figure 2).

In this study, the CO_2 concentration between day and night showed no significant differences ($p > 0.05$), which was proven by One-way ANOVA analysis, except between DCO_2S1 vs DCO_2S2 ($p = 0.014$, $p < 0.05$). Overall, based on analysis of 2-sample-t-test ($p = 0.303$, $p > 0.05$) and One-way ANOVA ($p = 0.294$, $p > 0.05$), no significant difference between CO_2 concentration during the daytime and night-time was shown.

Percentage of Soil Organic Matter (SOM)

Table 3 presents the *p*-value of SOM% among stations. There was no significant difference shows by SOM percentage between S1 vs S2 ($p = 0.918$, $p > 0.05$), while opposite results show by S1 vs S3 ($p = 0.001$) and S2 vs S3 ($p = 0.000$). No correlation coefficient shows between % SOM vs diversity indices (r = 0.348, $p = 0.774$), evenness (r = -0.289, $p = 0.813$) and richness (r = 0.972, $p = 0.151$) of dung beetles.

Table 3
Two-sample t-test analysis of SOM percentage (%) between Station 1, Station 2 and Station 3

p-value	S1 vs S2	S1 vs S3	S2 vs S3
% SOM	0.918	**0.001**	**0.000**

S=station; SOM=soil organic matter; Value in bold shows significant difference ($p < 0.05$)

DISCUSSION

In this study, Felda Lui Muda, an oil palm plantation located in the southern part of Peninsular Malaysia, was chosen as the model sampling site for its monocropping and nonnatural habitat. This was formerly recognized as a forest area, but it was transformed from a tropical forest habitat into a modified area because of its huge impact on the diversity and abundance of the different groups of dung beetle, most likely because it caused a rise in temperature (> 42 °C) during the day (Chown & Klok, 2011; Peyras et al., 2013). Therefore, the sampling sites of this study are the most suitable areas to measure the comparison. The baited pitfall traps, used in this study, were deemed suitable for use in the sampling activities, which involved active running insects on the soil surface, especially Carabidae and Staphylinidae. Chung (2004) had recorded a great diversity and abundance of Staphylinidae and Scarabaeidae, which were mainly dung beetles, at the ground level, justifying the use of the baited pitfall traps in this study.

In their study, Shahabuddin et al. (2005), found that the individual number of dung beetles in the agroforestry systems was the lowest, followed by young secondary forests and natural forest sites. The number of individual dung beetle samples collected from the natural forest sites (64.25 ± 9.12 samples) was three times higher than that in the agroforestry systems (22.75 ± 6.65 samples) and two times higher than that in the secondary forests with 26.25 ± 9.22 samples. Based on the assessment of the

dung beetle samples obtained from their study, the authors concluded that dung beetles were mostly scattered across various habitat types, with a high overlap between the same beetle species compositions among all the studied sites. According to Davis et al. (2002), forest destruction levels from primary, logged, and plantation showed a significant decline in dung beetle diversity in Northern Borneo. Both studies can be used to support this study which demonstrates the low diversity of dung beetles in agroforestry and plantation areas. Further research needs to be done to determine the type of the dung beetle species that can be found at both natural and plantation areas to support Shahabuddin et al., (2005) findings. For example, some species found in the current study, such as *O. orientalis*, *O. rugicollis*, and *O. rutilans*, were also collected at the Malaysian Production Forest (Yamada et al., 2014).

Soil organic materials and CO_2 concentrations are two new environmental factors that have been studied and measured in this research. The results of this study do not illustrate a correlation between the percentage of SOM and the diversity, evenness, and richness of the dung beetles. Similarly, they do not suggest a significant difference between the diurnal dung beetles and their nocturnal counterparts. As for the day and night CO_2 concentrations, however, the preliminary data on the parameters and the dung beetle abundance were successfully obtained. These two factors merit further investigation and comparison, as they are believed to significantly influence the behavior of the dung beetle species. Three parameters need to be studied to identify the effects on the presence of dung beetles: (a) the existence of nocturnal and diurnal species (Whitmore, 1990), (b) the exposure to CO_2 concentrations in the dung pads (Evans & Mamo, 2016), and (c) the richness of soil nutrients (Bang et al., 2005; Bornemissza & Williams, 1970).

In addition, Nichols et al. (2007) found no nocturnal species on open lands or modified forests. Thus, the question of the environmental factors and the diurnal and nocturnal species became interesting topics for further study. Moreover, other abiotic conditions, such as pH, temperature (Navarrete & Halffter, 2008; Peyras et al., 2013), and humidity (Vulinec, 2000) of the different ecosystems are strongly believed to affect the abundance and diversity of dung beetle species (Fincher et al., 1970). However, certain species such as *O. pacificus* had been found in agricultural areas, mainly because its distribution is not affected by habitat changes (Shahabuddin et al., 2005). According to Nichols et al. (2007), some small-sized species, such as *O. orientalis*, *C. unicornis*, and *O. proletarius*, are rarely found in the forest ecosystems but can easily be found in the open land areas; similar species were collected in this study.

In this study, the average CO_2 concentration during the daytime was slightly higher (329 ppm) than that at nighttime (321 ppm). This finding is congruent with Campbell et al. (2008), in that the CO_2 during the daytime was found to be higher than the nighttime. According

to Reece et al. (2011), $6CO_2$ and water have been used by plants to produce O_2 and organic matter through the process of photosynthesis with the presence of light. However, the Calvin cycle process occurs at night using $3CO_2$ as a source for converting to organic compounds without using direct light. This process is known as photorespiration, which involves the use of O_2 and the release of CO_2 with the presence of light.

The release of CO_2 in the oil palm plantations is influenced by autotrophs' respiration rates and the heterotrophic respiration of non-living organisms, such as soil decomposers (Koh et al., 2011). In addition, the sampling stations are located near the road, where the concentration of CO_2 in the area is affected by traffic, including the number of the vehicles present during the daytime, which is higher than the nighttime. In fact, the extent of CO_2 concentration in the air during the daytime (Station 2) may also be affected by its proximity to the swamp. In this study, the highest CO_2 concentration at night was recorded at Station 1, with only one ppm higher compared to Station 2. Further study should be conducted to determine the other biotic and abiotic factors contributing to the increment of CO_2 concentration in these sampling stations.

The results of this research illustrate that the abundance of *Onthophagus* sp. found in Stations 1 and 2 during the daytime is not significantly different ($p = 0.684$, $p > 0.05$), which contrasts the results of CO_2 concentrations. Hence, these data can be used to strongly suggest that the abundance and diversity of dung beetles are not influenced by CO_2 concentration in the environment. The CO_2 reading observed in this study is different from that recorded by Brooks et al. (1997), in that more CO_2 is released through respiration at night and no photosynthesis is performed by plants. Scientifically, atmospheric CO_2 concentrations may not affect the activities of the beetle species. More studies relating to CO_2 should be carried out, and additional sampling is required to obtain more samples of the beetles and more CO_2 data in the area.

The low O_2 or hypoxic condition in the dung pad is influenced by the content and concentration of CO_2 and methane (Scholtz et al., 2009), which will affect the ability of each species to survive at different CO_2 concentrations (Hoback, 2012). All beetle groups including roller, dweller, and tunneler are susceptible to hypoxic conditions at different times, depending on their behavior. When making balls from dung sources, adult dung beetles from roller group will be exposed to hypoxic conditions for several minutes (Tribe, 1976) or even hours (Osberg, 1988). Larger dung beetles are able to adapt and survive longer in hypoxic conditions compared to smaller dung beetles, mainly because of their ability to hold more air in the trachea (Hoback et al., 2000). Similar effects are expected to happen to dung beetle species if they are exposed to high CO_2 concentrations.

In this study, the impact of SOM, which forms part of the soil, on the diversity and abundance of *Onthophagus* spp. was

studied. The results illustrate no significant difference in the abundance of dung beetles across all the stations. However, S1 vs S3 and S2 vs S3 have significant differences based on the *p*-value being less than 0.05. According to Shannon's diversity index (*H*'), there is little difference in the indexes of the stations, suggesting that the percentage of SOM does not influence the dung beetle diversity index. It can be concluded that the abundance of *Onthophagus* spp. is inversely proportional to the percentage of SOM. When SOM contents increase, the abundance of *Onthophagus* spp. shows no significant difference across the stations. Taking the findings of SOM and the diversity and abundance of *Onthophagus* spp. into consideration, it can be concluded that SOM contents do not have a major influence on dung beetles' activities in the oil palm plantation study area.

The composition of the soil varies according to the type of soil, and the soil type selection by dung beetles depends on the species. The communities of dung beetle have been shown to vary according to sand and clay soil types. According to Silva et al. (2015), the physical and chemical properties of soil are able to influence the structure, diversity, and reproduction of dung beetles. In addition, the survival and reproductive success of certain dung beetle species can also be affected by soil properties, such as moisture content (Martinez et al*.,* 2009). A previous study found no difference in the overall abundance of dung beetle species, although larger paracoprid beetles tend to be dominant on sandy soils but not clay soils (Thurow et al*.,* 1986). Soil type also influences the survival of dung beetles, with beetle larvae remaining in the soil for weeks or months before emerging as adults (Halffter & Edmonds, 1982).

Humus is the major organic component of topsoil. Humus consists of organic material produced by dead organisms, fallen leaves, and feces decomposition. Other sources of humus include the decomposition of organic matter by bacteria and fungi. Some soil-inhabiting animals, such as earthworms, consume organic matter, excrete waste, move large amounts of material to the soil surface and move the organic matter into deeper layers of the soil (Campbell et al*.,* 2008). Dung beetles exhibit similar behavior. Based on the earlier works and the results of this study, it can be assumed that SOM is not a source of organic material to attract dung beetles, mainly because the adults and larvae from this subfamily are detritivores. They tend to use decaying organic materials for food by decomposing the mammal excrement, dead animal carcasses, and rotting plant matter (Halffter & Mathews, 1966).

In this study, the dung beetles exploited the organic matter in dung pads rather than SOM. In the study on dung beetle species' attraction to various types of bait, Goh (2014) found that most species (e.g., *O. babirusoides* and *O.vulpes*) that were active during the day were attracted to fish-baited traps and rat carcasses. In the same study, he found that small rollers tended to respond to dung-baited traps and that large rollers (e.g., *Paragymnopleurus*)

were common in both fish-baited and dung-baited traps. Furthermore, some species, including *Ochicanthon* spp., *Onthophagus* cf. "babirusa group" sp. Br, and *O. rudis*, selected traps that were baited with dead millipedes, despite the presence of traps baited with rotting fish. Thus, as evident in this study and those in the literature, SOM does not seem attract dung beetles.

CONCLUSION

In conclusion, although daytime and nighttime CO_2 concentrations demonstrated no remarkable differences, except between Station 1 and Station 2 during the daytime (DCO_2S1 vs DCO_2S2), this finding does not significantly influence the diurnal and nocturnal diel flight activity of the dung beetles or their abundance and diversity. In general, the abundance and diversity of *Onthophagus* spp. were similar across all test stations ($p > 0.05$), despite different percentages of organic materials; this demonstrates that SOM does not have a significant impact on *Onthophagus* spp. and CO_2 concentration. Additional research is needed to improve our knowledge of dung beetle diurnal species (e.g., *O. dayacus*, *O. denticollis*, *O.* cf. *pacificus*, and *Onthophagus* "hairy group") and nocturnal species (e.g., *O. babirussoides*, *O. phaenids*, *O. insicus*, *O. paraphamaeomorphos*, *O. peninsularis*, and *O. parachandrai*). Additional study should be conducted to advance our understanding of the relationship between dung beetles and their environment and especially of the effect of CO_2 concentration and soil organic materials.

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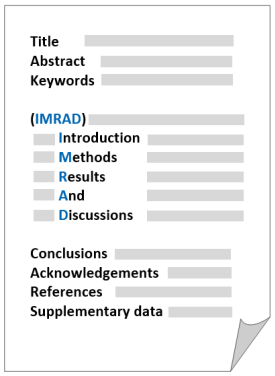

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