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Identification and Quantification of Cucurbitacins B and E in Different Parts of Bitter Gourd Plants Derived from Different Planting Methods

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ABSTRACT

Bitter gourd is a beneficial and easily accessible plant commonly utilised as a food source and medicinal herb. This plant produces numerous types of phytochemicals, especially when triggered by elicitors. It is also well known for its bitter taste, which is contributed by one of its phytochemical contents called cucurbitacin. This study determines the different levels of cucurbitacins B and E in the plants from two different planting methods, conventional and fertigation. Fruits, leaves, stems, and roots of bitter gourd plants from the two different planting methods were harvested for extraction using the sonication extraction method. The extraction solvents used were n-hexane, chloroform, and 80% ethanol. The extract's cucurbitacins B and E content were identified and quantified using high-performance liquid chromatography. A preliminary rapid test using the Salkowski's test to detect triterpenoids showed positive results for all sample runs. Results indicate significant variations in cucurbitacin levels across plant parts and cultivation methods. This study found that the content of cucurbitacin B in leaves of the fertigation planting method was the highest (208.0±0.4 ppm). Cucurbitacin B content in fruits was notably higher in both fertigation (200.0±1.3 ppm) and conventional (200.0±5.0 ppm) methods compared to other plant parts. However, leaves in the conventional method showed a significantly lower cucurbitacin B content (122.0±5.0 ppm). All plant parts were significantly different for cucurbitacin E, with the stem from the conventional planting method having the highest level

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of cucurbitacin E (31.0±1.0 ppm). Thus, it is concluded that plant parts and type of planting method can affect the cucurbitacin content in bitter gourd.

Keywords: Bitter gourd, cucurbitacin, fertigation, high-performance liquid chromatography, phytochemical

INTRODUCTION

Plants are a source of a large variety of organic compounds called secondary metabolites that have been utilised for a long time by human society. Secondary metabolites are complex compounds with unique carbon skeletons (Pagare et al., 2015). These compounds are present in all plant parts and involve various physiological processes as they can be synthesised in plant organs. They are not fixed to a single plant part but are defined by their biosynthesis and functions (Singh & Sharma, 2014). These processes are diverse and determined by cell type, environmental condition, and development stage (Kurepin et al., 2017; Li et al., 2020; Sanchita & Sharma, 2018).

Bitter gourd (*Momordica charantia*) is a food crop grown for food and medicinal purposes worldwide, especially in tropical regions. Bitter gourd is a family of Cucurbitaceae (Gayathry & John, 2022). According to studies by Jia et al. (2017) and Torre et al. (2020), bitter gourd produced a wide variety of secondary metabolites that give many beneficial properties, such as antioxidant, antimicrobial, anti-inflammatory, anticancer, and nutritional values. Aqueous, ethanolic, methanolic, and hexane extracts of bitter gourd contained alkaloids, glycosides, cholesterol, saponins, flavonoids, and terpenoids (Jia et al., 2017; Gayathry & John, 2022; Supraja et al. 2015). The primary constituents of this plant were cucurbitacins, sterols, saponin's cucurbitane-type triterpenoids (Chekka & Mantipelly, 2020), and vicine (Ahamad et al., 2017). Cucurbitacins are secondary metabolites found in many plant families but are highly common in the Cucurbitaceae plant family (Chanda et al., 2020; Haq et al., 2019). Cucurbitacin derivatives are differentiated by their structural skeleton and functional group arrangement (Haq et al., 2019). Cucurbitacins are known as the source of the cucurbitaceous plant's bitter taste (Kaushik et al., 2015). The cucurbitacin group currently consists of at least 19 members, which is differentiated by chemical structure variation between members named cucurbitacins A - T (Hunsakunachai et al., 2019). Similar to other cucurbitaceous plants, cucurbitacins are the main compound in bitter gourd (Maja et al., 2022).

Planting methods are one of the factors that affect the phytochemical accumulation and antioxidant activities in plants (Machado et al., 2018). The fertigation and raised bed (conventional planting) systems are two distinct growth conditions for plants, especially in media conditions. A fertigation system is a planting practice that allows precise and uniform amounts of water and nutrient supply to plants planted on non-soil or partial soil media through drips or sprinklers (Kant & Kafkafi., 2005). Raised bed planting is a planting technique on a bed above the existing soil level (Akbar et al., 2007). The raised bed is sometimes

covered with mulch to prevent water loss and suppress weeds. These two planting methods will give two distinct agronomic conditions affecting the plant's secondary metabolite (Neugart et al., 2018). Thus, different planting methods might stimulate the level of these factors that act as elicitors for secondary metabolite production.

From the understanding of the secondary metabolites synthesis mechanism and its beneficial effects on the *M. charantia* plant's defence system, the present project was planned to illustrate how the planting method impacted the production of the cucurbitacins in the bitter gourd plants.

MATERIALS AND METHODS

Plants Material

Seeds of bitter gourd were obtained from a local supplier. Seeds were soaked in water overnight to induce germination and sown in a germination tray filled with peat moss. After reaching the 3–4 leaf stages, the seedlings are transferred into a polybag prior to planting via conventional or fertigation systems. The planting beds were covered with silver shine and watered using a drip irrigation system for the conventional system. Plants in the conventional systems were fertilised manually with NPK fertiliser 15:15:15 one week after transplanting and NPK 12:12:17:2 on weeks 2, 5, and 8 at a rate of 30 g per plant (Anem, 2017). For the fertigation system, AB fertiliser was applied through a drip irrigation system (Kadir, 2020). There were 60 seedlings set up for each conventional and fertigation planting method. Both planting methods were conducted based on commercial practice described by Anem (2017) and Dhillon et al. (2017).

Preparation of Plant Extracts

The extraction method was conducted following the methods described by Attar and Ghane (2018), with slight modifications. Plant samples from both planting methods were harvested 60 days after sowing. Plant parts, including stems, roots, leaves, and fruits, were rinsed with distilled water, cut into small pieces and oven-dried at 50°C for 48 hr. The dried plant parts were then ground into powder. A total of 10 g of sample powder from each plant part was soaked into 100 ml of n-hexane (Merck, Germany). The mixture was placed in an ultrasonic bath (Elma Ultrasonic Cleaner 15 L Digital Ultra Sonic Tank Bath Cleaning Heater Timer PS-60, Germany) at 35°C for 40 min. The extract was filtered through a filter paper (Whatman™ 1001-125 Grade 1 Qualitative Filter Paper, diameter: 12.5 cm, pore size: 11 µm, USA). The filtrate was centrifuged (Hitachi, Japan) for 15 min at 905.58 g and filtered through the filter paper. The filtrate was re-extracted using chloroform (Thermo Fisher Scientific, USA) and 80% ethanol (Merck, Germany) following the n-hexane steps. The 80% ethanol (Merck, Germany) extraction filtrate was dried under vacuum at 40–60°C using a rotary evaporator (R-215, BUCHI, Switzerland).

Terpenoid Qualitative Rapid Screening (Salkowski's Test)

Qualitative phytochemical screening for steroids and triterpenoids in bitter gourd

plant parts was done using the Salkowski's test. A total of 1 g of plant extracts was exposed to a few drops of chloroform (Thermo Fisher Scientific, USA) and concentrated sulfuric acid (Grainger, USA) and let rest. After several minutes, a lower layer with a red-to-yellow colour indicated the presence of steroids and triterpenoids (Ramakrishna et al., 2019).

Standard Preparation

A total of 1 mg of each cucurbitacins B and E standard compound powder was dissolved in 1 ml acetonitrile (Merck, Germany) (stock solution). Further dilution was done by diluting the standard stock solution with acetonitrile to prepare a concentration range of 0.1–1.0 mg/ml for both standards. The standard solutions were filtered through a 0.45 µl syringe filter before injected into high-performance liquid chromatography (HPLC, Shimadzu/LC 20AT, Japan) and analysed to plot a standard curve. Both compounds from bitter gourd extracts were then identified and quantified by comparing them with the standards' standard curves and retention time.

HPLC Condition and Analysis

Compounds in the extract were identified and quantified using HPLC (Shimadzu/LC 20AT, Japan), following the method described by Zaini et al. (2018) with slight modifications. A total of 1 mg of each extract was dissolved in 1 ml of acetonitrile (Merck, Germany). The solution was filtered through a 0.22 µm syringe filter and pumped through the Syncronis™ C18 column (250 mm x 4.6 mm diameter, Thermo Fisher Scientific, USA). The flow rate and wavelength were optimised at 1 ml/min and 250-280 nm, respectively. A total of 2% acetic acid (Merck, Germany) in water (solvent A) and acetonitrile (Merck, Germany) (solvent B) were used as mobile phases with a gradient elution system. A 0.45 µm Millipore™ (Merck, Germany) membrane was used to filter the mobile phase, and the degas were filtered using sonication. The column temperature was maintained at 30°C, and the sample injection volume was set to 20 µl.

Data Analysis

The data obtained were analysed using analysis of variance (ANOVA). The means and standard deviation for the cucurbitacins B and E quantities were determined and compared using the Tukey test to find the significantly different mean within the same planting method, at the 5% significance level.

RESULTS AND DISCUSSION

Salkowski's Test

The Salkowski's test is a qualitative phytochemical assessment generally for terpenoids and their isomers, including tetracyclic triterpene cucurbitacins (Sasikala & Sundaraganapathy, 2018). This study used the Salkowski's test, a simple and supportive technique to detect triterpenoids. Crude extract of each bitter gourd plant part from both planting methods showed positive results, as presented in Figure 1. It indicated that triterpenoids and their isomers are present in the crude extracts

of all bitter gourd plant parts from both planting methods (Ramakrishna et al., 2019). It supports the assumption that cucurbitacins are present in the crude extract and demonstrates a promising result for HPLC analysis. All plant parts of bitter gourd contain terpenoids, including triterpenes's cucurbitacins (Ramakrishna et al., 2019). Thus, it is assumed that triterpenoids of cucurbitacins were among the detected terpenoids (Kaushik et al., 2015). The difference in intensity of the red-brownish colour that appeared might be affected by the concentration of the phytochemical compound (terpenoids). In the Salkowski's test, terpenoids are regarded as steroids due to their 5-5-carbon ring structure (Adedokun et al., 2023). The colour changes due to the reaction between sulphuric acid and the aromatic rings of steroids (Gupta, n.d.). It indicates that the reaction was straightforward and continues to occur if there are reagents. The chemical reaction might increase with increasing reagents (phytochemical and solvent concentration) (Key, 2014). However, despite the intensity of the colour formed, the criteria to accurately acknowledge the presence of the target compounds are only based on the empirical positive or negative result (Maharaj et al., 2017; Pochapski et al., 2011). Figure 1 shows that the stem extract from the fertigation method contains the highest terpenoid, including its derivative concentration, while the extract from the conventional method contains the lowest terpenoid concentration and its derivative.

Quantification of Cucurbitacins B and E

The standard curve R^2 values of 0.9986923 and 0.9961842 were obtained from the calibration curves of the cucurbitacins B

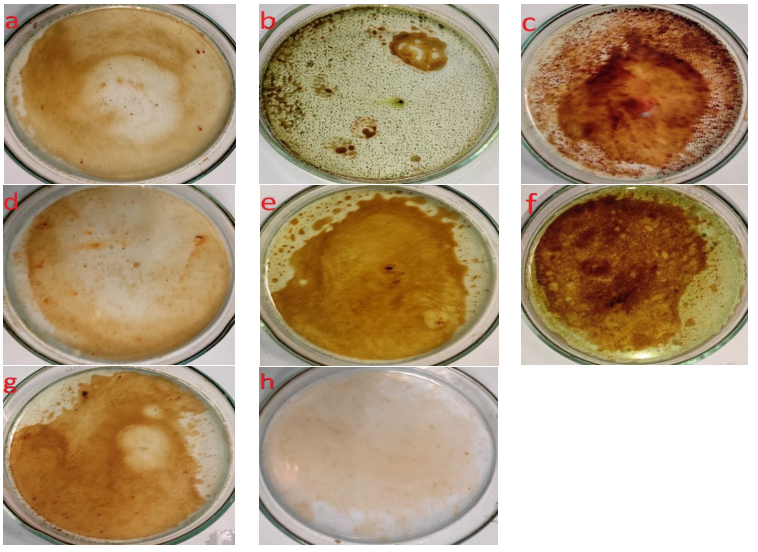

Figure 1. Phytochemical screening using the Salkowski's test for terpenoids of different plant parts of bitter gourd. The formation of a lower layer with a red to yellow colour indicates the presence of steroids and triterpenoid in (a) fruits from fertigation method, (b) leaves from fertigation method, (c) stems from fertigation method, (d) roots from fertigation method, (e) fruits from conventional method, (f) leaves from conventional method, (g) stems from conventional method, and (h) roots from conventional method, respectively

and E standard compounds, respectively. The levels of cucurbitacins B and E were compared between plant parts from the same propagation method and between the same plant parts from each of the different propagation methods.

For the fertigation planting method (Table 1), leaves contain the highest content of cucurbitacin E at 13.0±7.6 ppm and cucurbitacin B at 208.0±0.4 ppm. On the other hand, fruits have the lowest cucurbitacin E content at 2.0±0.6 ppm and the second highest cucurbitacin B content at 200.0±1.3 ppm. Cucurbitacins B and E content in stems is 185.0±0.4 ppm and 11.0 ±1.5 ppm, respectively. While cucurbitacins B and E in roots are 174.0±0.6 ppm and 5.0 ±0.0 ppm, respectively. It can be observed that the cucurbitacin B content in leaves of fertigation plants was significantly higher than that of stems and roots but not significant in fruits. For cucurbitacin E, leaves were only significantly higher than fruit, and there was no significant difference between fruits, stems, and roots.

For the conventional method, stems contain the highest content of cucurbitacin E at 31.0±1.7 ppm but the second lowest cucurbitacin B at 136.0±4.5 ppm. Fruits came with the highest content of cucurbitacin B at 200.0±5.0 ppm but also with the lowest content of cucurbitacin E at 5.0±0.1 ppm. Cucurbitacins B and E content in leaves was 122.0±10.5 ppm and 13.0±0.1 ppm, respectively. The root contains 9.0±1.2 ppm of cucurbitacin E and 166.0±2.5 ppm of cucurbitacin B. Analysis of variance showed that the content of cucurbitacin B in fruits was significantly higher than all other plant parts from the same planting method. All plant parts were significantly different for cucurbitacin E, with the stem having the highest level. The level of cucurbitacin B in all plant parts from both planting methods is higher than cucurbitacin E content. For comparison between planting methods, all plant parts from the fertigation method contain a higher level of cucurbitacin B compared to plant parts from the conventional method. However, only leaves and stems differ significantly.

Fruits and stems from the conventional planting method contain higher cucurbitacin E compared to fruits and stems from the fertigation method and are significantly different. Leaves from both planting methods are equal and not significantly different. At the same time, cucurbitacin E in roots from the conventional planting method is lower than in the fertigation method, and there is no significant difference either. Sikander et al. (2019) stated that cucurbitacins B and D are the most common cucurbitacins to be found in plants. Cucurbitacin B is also the highest contributor to the bitter taste in bitter gourd, while other cucurbitaceous plants with cucurbitacins combination (Luo et al., 2020; Maja et al., 2022).

Previous studies have also indicated that abiotic stress may increase the production of secondary metabolites while the crops are growing. *Cucumis prophetarum* callus cultured in media with sodium chloride as an imitation of salt stress contains a higher level of cucurbitacin compared to the absence of sodium chloride (Saker et al., 2010). The level of cucurbitacin E was also

recorded to be higher in fruits, leaves and roots of cucumber plants that were treated with potassium phosphate and chitosan as signalling compound elicitors compared to control with fruit has the highest content of cucurbitacin (Ramezani et al., 2017).

Overall, the content of cucurbitacin B in plant parts cultivated through fertigation and conventional methods exhibited variation, as statistically significant differences were observed. Furthermore, stems and roots from the conventional planting method demonstrated higher cucurbitacin E content compared to the fertigation method, whereas fruits and leaves showed similar levels. Notably, the highest content of cucurbitacin B was recorded in the leaves of the fertigation planting method. However, this difference was not deemed statistically significant compared to fruits from the fertigation and conventional planting methods. Similarly, the highest concentration of cucurbitacin E was observed in stems from the conventional planting method, with a significant difference noted compared to all other plant parts from both planting methods. No specific studies on the cucurbitacin B content of bitter gourd have been identified in previous research. However, Maja et al. (2022) reported cucurbitacin B content in the plant parts of various species. The leaves extract of *Citrullus lanantus* var. *lanantus* showed 0.3 ppm, *Citrullus colocynthis* exhibited 20.8 ppm (Kim et al., 2018), *Cucumis africanus* presented 0.082 ppm (Shadung & Mashela, 2016), and *Lagenaria siceraria* recorded 0.05 ppm (Mashilo et al., 2018). Furthermore, *Cucumis melo* displayed 65-75 ppm and 70-80 ppm in leaves and fruit extracts, respectively (Luo et al., 2020). Da Rocha Galucio et al. (2022) identified 79 ppm of cucurbitacin B in the ethanolic extract of *Luffa operculata* (L.) Cogn. fruits, considering it a substantial amount. It is noteworthy that the cucurbitacin B content reported in these previous studies was lower than the highest content observed in the present study. Contrastingly, for cucurbitacin E, previous studies present results opposite to the highest cucurbitacin E recorded in the present study. Chanda et al. (2019) extracted 356 and 663 ppm of cucurbitacin E from bitter gourd and *Cucurbita pepo* var. *pepo* fruits, respectively, higher than observed in the present study. The decrease in cucurbitacin E content in the present study is assumed to be caused by the modification of cucurbitacin E during the production of other cucurbitacin derivatives. Cucurbitacins B and E are the primary cucurbitacins that can be modified through the action of acetyl esterases into other cucurbitacins (Ahmed & Halaweish, 2014; Schabort et al., 1968).

The variation of cucurbitacins B and E level in different plant parts of the bitter gourd samples is consistent with the study by Kim et al (2018) and Luo et al (2020). Different concentrations of cucurbitacins were also recorded in other species of cucurbits (Maja et al., 2022). It is due to the differences in type and level of stress from the surrounding environment faced by each of the plant parts, even for the same individual plant, as the production of secondary metabolites is specifically based on organ, tissue, and even cell of the plants (Pagare et al., 2015; Ramezani et al.,

2017). Environmental changes can have a significant impact on the regulation of secondary metabolic processes (Sanchita & Sharma, 2018). Different plant sections have varied gene expression patterns associated with cucurbitacin production (Luo et al., 2020; Maja et al., 2022). Maja et al (2022) also suggested that different cucurbitacins have different cucurbitacins synthesis genes and gene expressions. It demonstrated that the level of cucurbitacins B and E recorded cannot solely be used to support the hypothesis that cucurbitacin content increases with stress. The production of secondary metabolites in plants is complex because other types of cucurbitacins may be produced during the stress, as the synthesise of cucurbitacins and other secondary metabolites is also genotype-dependent (Luo et al., 2020; Maja et al., 2022). Once elicitors, including abiotic stresses, are recognised by plants, the manufacture of genetic transcription factors that control plant secondary metabolism type and function will be initiated (Ramezani et al., 2017).

Table 1
Cucurbitacins quantity of bitter gourd plant parts

Phytoconstituent (Cucurbitacins)	Method	Quantity of cucurbitacins (ppm)			
		Fruits	Leaves	Stems	Roots
Cucurbitacin B	Fertigation	$^{ABa}200.0\pm1.3^{w}$	$^{Aa}208.0\pm0.4^{w}$	$^{BCa}185.0\pm0.4^{w}$	$^{Ca}174.0\pm0.6^{w}$
	conventional	$^{Aa}200.0\pm5.0^{w}$	$^{Bb}122.0\pm10.5^{x}$	$^{BCb}136.0\pm4.5^{x}$	$^{Ca}166.0\pm2.5^{w}$
Cucurbitacin E	Fertigation	$^{Aa}2.0\pm0.6^{x}$	$^{Ba}13.0\pm7.6^{y}$	$^{ABa}11.0\pm1.5^{y}$	$^{ABa}5.0\pm0.0^{x}$
	conventional	$^{Ab}5.0\pm0.1^{x}$	$^{Ba}13.0\pm0.1^{y}$	$^{Cb}31.0\pm1.7^{z}$	$^{Da}9.0\pm1.2^{x}$

Note. Different ABCD letters are significantly ($p < 0.05$) different data between plant parts; Different abcd letters are significantly ($p < 0.05$) different data between planting methods; Different wxyz letters are significantly ($p < 0.05$) different data between cucurbitacins on plant parts for both planting methods

CONCLUSION

This study reveals that plant parts and type of planting method can affect the cucurbitacin type and content in plants. This study also supported previous studies that cucurbitacin B is the common cucurbitacin in bitter gourd plants compared to cucurbitacin E. Type and imposition of elicitors and plant tissues are assumed to play an important role in defining the level and type of secondary metabolites, especially cucurbitacins in this study. This research sheds light on the interplay between planting methods, plant sections, environmental stresses, and secondary metabolite synthesis, allowing for a more nuanced understanding of cucurbitacin concentration differences in bitter gourd samples. Further analysis and experimentation would be needed to understand the underlying factors driving these differences in cucurbitacins B and E content between planting methods and plant parts.

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