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RESEARCH ARTICLE

Morphological Analysis and Comparison of Asiaticoside Content in *Centella asiatica* Grown in Different Regions of Malaysia

Nur Hazirah Zainal[1], Hannis Fadzilah Mohsin[2], Zolkapli Eshak[2] and Noor Anilizawatima Sulong[1*]

[1]Faculty of Pharmacy, Universiti Teknologi MARA (UiTM) Selangor Branch, Bandar Puncak Alam - 42300, Selangor, Malaysia; watima@gmail.com

[2]Department of Pharmacology and Pharmaceutical Chemistry, Faculty of Pharmacy, Universiti Teknologi MARA (UiTM) Selangor Branch, Bandar Puncak Alam – 42300, Selangor, Malaysia

Abstract

Background: *Centella asiatica* L. Urban is a medicinal plant that contains Secondary Metabolites (SMs) such as terpenoids that not only function in environment adaptation but importantly give potential wide medicinal value specifically asiaticoside such as brain tonic and anti-inflammatory. This very reason causes a rise in market demand which may lead to plant overexploitation considering that the plant grows very slowly in natural settings and only produces a small amount of SM. Previous studies have reported a few factors including soil composition, lighting and temperature that may affect the growth of the plant and its SM production. **Aim:** This study aimed to compare the asiaticoside accumulated in *C. asiatica* grown in different regions and the factors that may affect its accumulation. **Methods:** In this study, the High-Performance Liquid Chromatography (HPLC) method was used to identify and compare the asiaticoside content in *C. asiatica* from Sungai Buloh, Bukit Cerakah and Sungkai and the concentration was then calculated by utilisation of calibration curve. Field Emission Scanning Electron Microscopy (FESEM) was used to analyse the plant cell morphology and pH measurements of soil were conducted. **Results:** The crude extract obtained from *pegaga* of Sg. Buloh with clayey and acidic soil exhibited the highest asiaticoside content followed by Bukit Cerakah and Sungkai. Other factors such as the optimal light intensity, photoperiod, temperature, soil fertility and soil water status were also key factors that significantly enhanced the asiaticoside production. **Conclusion:** This study demonstrates that distinct growing conditions particularly the type and acidity of soil play a vital role in the production of SM. However, further studies are necessary to explore the soil nutrients and the simultaneous effects of multiple environmental factors on the biosynthesis of SM to ensure favourable plant cultivation and maximise SM production.

Keywords: Environmental Factors, Medicinal Plant, Quantification, Secondary Metabolite, Terpenoids

1. Introduction

Centella asiatica L. Urban is a plant family of apiaceae which is known as Asiatic Pennywort or *gotu kola*[1,2]. It is native to Southeast Asian countries including India as practised in Ayurvedic medicine, China as folk medicine practice and Malaysia commonly called by the natives *pegaga* whether it is consumed as herbal remedies or as *ulam* (salad) interpreted more commonly as traditional vegefig taken in a meal among the Malay community[3].

Morphologically, the plant can be described as a small, perennial creeping herb with shovel-shaped green leaves and internode root[2]. The stem is slender, creeping stolons with shallow grooves and appears reddish-green or brownish[2]. This plant is tasteless and due to the presence of essential oil or resin, it has an aromatic fragrance[2]. The phytochemical constituents

*Author for correspondence

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found in the plant not only serve as the plant defence mechanism or adaptation to abiotic stress but also give about variety of valuable medicinal benefits[4].

Studies on *C. asiatica* have long been started and up to this day, around 139 secondary metabolites have been reported to be extracted from it[5]. These include triterpenoids, phenolic acids, flavonoids, glycosides, fatty acids and many others[1,2,5]. Along with saponins, madecassoside and madecassic acid, asiaticoside and Asiatic acid are the most plentiful compounds found in the *C. asiatica* under the group of triterpenoids which is biosynthesised via the isoprenoid pathway that starts with the isomeric 5-carbon precursors, isopentenyl diphosphate and dimethylallyl diphosphate[6]. The assemblement of the C5 precursors by prenyl transferases will lead to the production of farnesyl diphosphate[6]. Then, the squalene synthase will catalyze the condensation of farnesyl diphosphate molecule to yield squalene which is then will be converted to 2,3-oxidosqualene by squalene epoxidase in the presence of oxygen (O2) and Nicotinamide Adenine Dinucleotide Phosphate (NADPH)[6]. The cyclization of 2,3-oxidosqualene is facilitated by the alpha-amyrin synthase hence, yielding triterpenoids such as asiaticoside and asiatic acid[6,7].

Sun *et al.*,[8] reported that the asiaticoside can reach its maximum levels within 5-15 minutes after it is administered orally and will be extensively distributed in the brain, stomach and skin within one hour[8]. Due to its glycosidic structure, this metabolite is soluble in water. Meanwhile, aglycones which are asiatic acids are usually insoluble in water[8] owing to their structure that is missing glycosides. This secondary metabolite is reported to be mainly absorbed in the jejunum[8,9] before it is distributed in the plasma, brain, heart, kidney, liver, colon and bladder followed by metabolism in the intestine[8]. Both of these metabolites are not only significant for plant defence mechanisms but, also harbour numerous pharmaceutical values[10].

Traditionally, through ingestion of the raw plant, a decoction of the root part as a tea or made into poultice or paste for external application, the wide medicinal value of *C. asiatica* includes its utilisation to treat gastrointestinal disease, as anti-diabetes, anti-inflammatory, mental rejuvenator, memory enhancer and many others[5,11] are owed to its phytochemical constituents.

Regarding asiaticoside, many studies have reported the potential therapeutic uses of this constituent and one of the most popular is what makes the plants to be considered as the brain tonic[5]. This is because the content of Brain-Derived Neurotrophic Factor (BDNF) may be increased effectively by this secondary metabolite[8,12]. A study conducted in rats by Sun and colleagues[8] found that asiaticoside may decrease the inflammatory factors, balance the oxidative stress, repair abnormal expression of mitochondrial-related proteins and improve the content of BDNF and thus, improve the neurological diseases[8]. Besides, these constituents may improve the survival rate of neural cells by reducing the related nerve cell apoptosis and increasing the synaptic density[13,14].

When undergoing hydrolysis, asiaticoside may be converted into its aglycone form in which the asiatic acid plays a role in repairing the oxidative stress damage caused by obesity in addition to promoting fatty acid oxidation to suppress weight gain in rats[1,8]. Besides that, both metabolites are found to be effective as direct wound healers such that they promote the process of repairing wounds by inducing the proliferation of fibroblasts and collagen synthesis moderately in rabbits[15,16], in addition to having an anti-bacterial properties and zero irritating side effect on the skin[8].

Given that secondary metabolites have become a crucial source to treat numerous human diseases and ailments whether traditionally or in the nutraceutical formulation, the market demand has been rising. This may lead to overexploitation of the plant since this naturally grown plant exhibits a very slow growth and only a small amount of secondary metabolites are produced in the whole plant[5] in addition to the time-consuming process of the active constituents extraction[10].

In the hope of combating this problem, researchers have spent decades to find ways to intensify the crop yields. Many studies have reported the factors that affect plant growth and its secondary metabolite accumulation. By taking into account the plant defence system against stress such that secondary metabolites including terpenoids may act as thermoprotectants[4] to regulate the plant temperature during heat waves, the other stimulations may also lead to the increase of plant secondary metabolism. This includes the stimulation of nutrients through abiotic elicitor[8], soil composition

used[17] and changes in environmental conditions such as temperature, lighting[18], ozone, soil water status, soil fertility and salinity[19,20]. In response to environmental changes, the type and amount of secondary metabolites regulated may be redirected[10] and such response may vary between plant species and phytochemical components[10].

Therefore, this study was conducted to observe the cell morphology of *C.*asiatica and to compare the amount of secondary metabolite, specifically asiaticoside between the three samples about their growing condition. This study can significantly serve as a guide towards a better understanding of possible factors that may optimise plant cultivation and hence, improve plant secondary metabolite production especially the asiaticoside in *C. asiatica*.

2. Materials and Methods

2.1 Materials

The plant materials (*C. asiatica*) along with its soil were obtained from three different origins namely Sungai Buloh Flowers and Plants Nursery (Sg. Buloh), Bukit Cerakah residence (Bukit Cerakah), Selangor and the one that grew wildly in Sungai Klah hot spring in Sungkai area (Sungkai), Perak, Malaysia. On the other hand, the centelloside for reference which is asiaticoside was purchased from ChemFaces. HPLC-grade methanol and acetonitrile were purchased from Sigma-Aldrich. Besides that, the buffer solution was freshly prepared by mixing the orthophosphoric acid obtained from the previous source together with purified water to obtain the solution of 0.3 per cent orthophosphoric acid.

2.2 Preparation of Plant Extract

The collected plants were cleaned thoroughly with tap water to prevent soil contamination before they were dried in the oven for three days at 35-40 °C. Then, they were ground with a laboratory blender. After that, following the cold extraction method, the ground plantlets were soaked in methanol successively at room temperature under dark conditions. The samples were kept for one week and were stirred for one day using the orbital shaker. Next, the samples were filtered before they were evaporated under reduced pressure with the aid of a rotary evaporator until greenish-brown crude extracts were obtained[21]. The crude extracts were kept in the fume hood until dried and kept at -20 °C until sample solution preparation was carried out.

2.3 Preparation of Samples Extracts and Reference Solution

After the evaporation process was carried out, the extracts were weighed and diluted in HPLC-grade methanol to form a 500 mg/L extract solution. The extracts were fully dissolved in the diluent by sonication and then filtered through a 0.45-micron Gelman filter to eliminate any impurities or insoluble compounds before being placed into the HPLC vials. Then, the reference substance for asiaticoside was separately weighed and solubilised in the HPLC grade methanol each. A 1 mg/mL reference stock solution was prepared, in which serial dilutions were performed next to obtain reference solutions of 1.000, 0.500, 0.250, and 0.125 mg/mL and thereafter reference solution of each concentration was syringed out and filtered through a 0.45 µm pore size filter into an HPLC vial.

2.4 High-Performance Liquid Chromatography (HPLC) Method

During the methanolic crude extraction, the extraction obtained consisted of asiaticoside which was isolated from the dried plantlets of *Centella asiatica* grown from different areas. Before the commencement of chromatographic techniques, the methanolic crude extracts were dissolved in absolute HPLC-grade methanol. After that, they were filtered using a 0.45 µm filter which only then they were injected into the Agilent HPLC 1200 Series system. For all sample solutions, reverse phase column chromatography (Hypersil GOLD Column; 4.6 mm x 250 mm) at a concentration of 5 mg/mL was utilised and eluted with buffer (0.3% orthophosphoric acid) to acetonitrile ratio as the mobile phase using the gradient elution program (Table 1) for method development. HPLC-MWD was performed under the following conditions: 1 mL/min; column temperature of 25 °C; Agilent Multiple Wavelength Detector (MWD) 1100; evaporator temperature of 40°C; N_2 flow rate at 1.6 L/min; injection 30 µL.

For the determination of asiaticoside retention time, the reference asiaticoside was dissolved separately in HPLC grade methanol as reference solutions with the previously stated concentration before it was injected into the HPLC. By comparing the retention time of

Table 1. Mobile phase (gradient mode): buffer to acetonitrile composition for asiaticoside analysis

Elution time (minute)	Buffer: Acetonitrile (%)
0.01	95: 5
5.0	80: 20
15.0	50: 50
20.0	20: 80
23.0	20: 80
30.0	50: 50
35.0	80: 20
40.0	95: 5

the chromatogram obtained for standard and sample solutions, asiaticoside from the methanolic crude extract was identified before quantification. The concentration of the asiaticoside obtained from the crude extract was then calculated with the utilisation of a simple calibration curve of a range of standard solutions where the peak area, and milli Absorbance unit (mAu) from the chromatogram were correlated to concentration.

2.5 Field Emission Scanning Electron Microscopy (FESEM) Analysis

The plant cell morphology such as its leaves, stem and root parts was observed using the FESEM under different magnifications such as 100x and 400x. This imaging technique was employed to observe the cells of the plant such as the xylem and stomata.

2.6 pH Measurement of Soil

Soil slurry of each soil from different areas was prepared by mixing 10 g of the soil with 25 mL of HI7051 soil sample preparation solution in a beaker vigorously for 30 seconds[22]. After five minutes of waiting, the pH electrode was immersed into the prepared sample and was swirled gently[22]. The measurement was recorded after the reading was stabilised.

3. Results

3.1 Morphological Analysis (FESEM)

As shown in Figure 1, some of the stomata of plants from all regions were observed to be opened. However, the stomatal opening in *C. asiatica* of Bukit Cerakah was wider compared to the plant of Sg. Buloh and Sungkai were only partially opened.

Meanwhile, from Figure 2, the xylem was observed where the xylem of *C. asiatica* from Sg. Buloh was thicker than the other two regions.

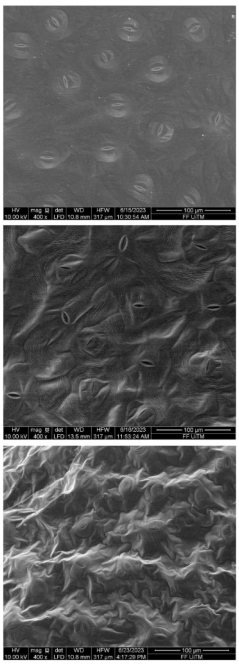


Figure 1. Abaxial surface of *C. asiatica* leaves observed under FESEM 400x. From top to bottom: Sg. Buloh, Bukit Cerakah and Sungkai.

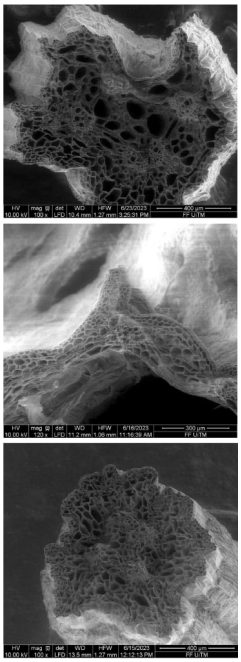


Figure 2. Cross section of *C. asiatica* stems observed under FESEM 100x. From top to bottom: Sg. Buloh, Bukit Cerakah and Sungkai.

Table 2. Average pH reading of each soil

Soil	Average pH
Sg. Buloh	3.78
Bukit Cerakah	6.57
Sungkai	6.79

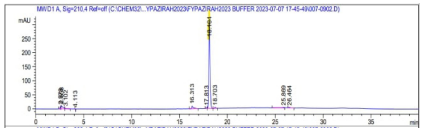


Figure 3. Chromatogram of asiaticoside standard solution 0.5 mg/mL.

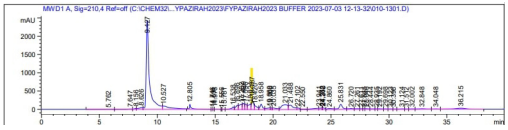


Figure 4. Chromatogram of leaves and stems crude extract of *C. asiatica* from Sg. Buloh.

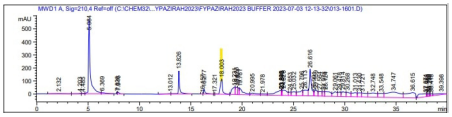


Figure 5. Chromatogram of roots crude extract of *C. asiatica* from Sg. Buloh.

3.2 Soil pH Measurement

As depicted in Table 2, the soil from Sg. Buloh gave more acidic conditions (pH 3.78) than Bukit Cerakah and Sungkai where both of them were only slightly acidic.

3.2.1 HPLC Analysis

The chromatogram between the standard solution (Figure 3) and sample solution of leaves and stem (Figure 4) and roots (Figure 5) were compared and the retention time obtained was around 18 minutes. After plotting the simple linear curve (Figure 6) and the peak area (mAu) that was correlated to concentration, the concentration of asiaticoside derived from *C. asiatica* from the three different regions was obtained as shown in Table 3.

The crude extracts of *pegaga* from Sg. Buloh gave the highest content of asiaticoside for both leaves and stems and root parts which is 0.6286 and 0.3431 mg/mL respectively, followed by that of Bukit Cerakah. On the other hand, the lowest asiaticoside content from both parts was *pegaga* from Sungkai.

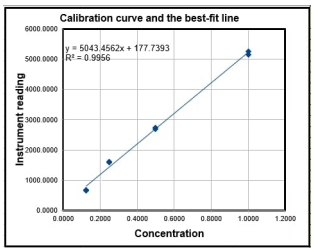


Figure 6. Calibration curve of asiaticoside.

Table 3. Calculated concentration of asiaticoside from each plant part of each location

Location	Calculated concentration (mg/mL)	
Plant part:	Leaves and stems	Roots
Sg. Buloh	0.6285	0.3431
Bukit Cerakah	0.4751	0.3184
Sungkai	0.2730	0.2038

4. Discussion

One of the environmental factors that may contribute to the production of secondary metabolite is light intensity[18-20]. The stomatal opening may occur when a plant is exposed to light, allowing the uptake of carbon dioxide (CO_2) to carry out photosynthesis[23]. Through photosynthesis, plants obtain energy to produce several carbohydrate molecules that are then utilised for plant metabolism and the generation of primary and secondary metabolites[4]. Meanwhile, closure of stomata may occur as a result of water stress as the plant adapts to limit water loss through diminished transpiration[24].

As suggested in Figure 1, the stomatal opening in *C. asiatica* of Bukit Cerakah was wider compared to the other two regions where only spatial opening can be seen. This may be due to the condition where the plant is grown directly under the sunlight without any shade in Bukit Cerakah unlike in Sg. Buloh and Sungkai where the plant is grown under the canvas or canopy shade in the nursery and surrounded by taller trees respectively cause quick fluctuation in light intensity which may lead to a lower average rate of photosynthesis[25]. Although only spatial stomatal opening can be observed, it may not be an indicator of water stress in both *pegaga* from Sg. Buloh and Sungkai as it was reported that partial closure of stomata only has a little effect on transpiration[26] given that both soil conditions are well-watered.

Albeit the wider opening of stomata in the Bukit Cerakah plant due to direct sunlight exposure, the centelloid obtained from both plant parts were lower compared to the content in *C. Asiatica* of Sg. Buloh where the plant is located under canvas protection. These findings are inconsistent with studies by Devkota and friends[27] and Alqahtani *et al.,*[28] that concluded the triterpene and flavonoid levels were higher in plants growing in full sunlight than in partial shade[27,28]. As mentioned before, the plant in Sg. Buloh is shaded by canvas. This may cause the availability of Photosynthetically Active Radiation (PAR) for photosynthesis to be affected[29] and the same situation may be applied to Sungkai where the plant grown is shaded by bigger trees. Outside of the optimum range of PAR intensity may lead to photoinhibition and disruption of photosynthetic apparatus[29,30]. Given that the asiaticoside content of *pegaga* in Sg. Buloh was the highest, the light intensity received by the plant chlorophyll may be sufficient for the plant photosynthesis in addition to the duration of the plant introduced to the light (photoperiod) may also noticeably influence the biosynthesis of secondary metabolites. For example, studies reported that a longer photoperiod may improve the phenols accumulation[10,31] and thereby, the photoperiod may have compensated for the fluctuated PAR intensity received in Sg. Buloh case.

One thing to be noted here is that the observation of plant cell morphology under FESEM for each plant was taken at different times (Sg. Buloh: 10:30 A.M; Bukit Cerakah: 11:53 A.M; Sungkai: 4:17 P.M) due to equipment unavailability and understaffing issues. So, as the PAR intensity fluctuates within a day[29], the light-induce stomatal opening may be affected such that the light intensity is lower in the morning, increased when approaching noon and declined after 3 in the afternoon similar to the report by John and Peirce[32] in which the increase and decrease of sunlight during the day affects the width of stomatal opening.

But, taking photoperiod and light intensity into consideration, the *C. asiatica* of Sg. Buloh received lower PAR intensity than in Bukit Cerakah and may be similar in Sungkai but still regulates the highest active metabolites. So, the reason underlying may be the consequences of another rate-limiting factor that limits photosynthesis. To carry out photosynthesis, the process can be divided into two phases which are the light-dependent reaction and light-independent reaction. Involving light-dependent reaction, as light intensifies, the rate of photosynthesis increases, generating more adenosine triphosphate (ATP) and NADPH necessary for asiaticoside (secondary metabolite) synthesis[33,34]. In a condition where very high PAR intensity, a photosynthetic rate may eventually drop as a result of chlorophyll being damaged due to receiving light more than it can utilise[29,33]. So, being exposed to direct sunlight, too much light intensity may lead to the decline in photosynthesis of *C. asiatica* from Bukit Cerakah and thus, lower asiaticoside biosynthesis than that of Sg. Buloh. However, both *pegaga* from Sg. Buloh and Sungkai were under shade, lowering the PAR intensity, but the centalloids obtained were the highest for the former but lowest for the latter.

This is when other photosynthesis rate limiting factors might play their role. In light-dependent reactions, as the light intensifies, the rate of photosynthesis increases until it reaches a plateau due to another factor. Relating to the light-independent reaction of photosynthesis, temperature might influence it although it may not affect the light-dependent reaction[33]. Photosynthesis is catalysed by enzymes where elevated temperature will improve the overall rate[33]. This is in parallel with a few studies that concluded the higher temperature will lead to higher secondary metabolites content such as alkaloids and terpenoids[4,10]. The capacity for isoprene emission was also found to be doubled in a study conducted on *Quercus alba* and *Q. rubra* when taken care of in warmer condition[35] and this may also be correlated with the asiaticoside generation of *C. asiatica* since both compounds are under the same class and follows the similar isoprenoid pathway.

However, too high a temperature may lead to the denaturation of enzymes needed for photosynthesis[33]. Concerning this study, although shade does not necessarily cool the temperature, direct sunlight exposure such as in the case of Bukit Cerakah may cause the area to be warmer than in Sg. Buloh. This is because direct sunlight rays carry energy that will be absorbed and converted into heat once it reaches a surface making it feel warmer than those under the shade[36]. The warm condition that is higher than the optimum temperature may cause lower production of secondary metabolites as suggested in a study conducted on

Zea mays by Christie *et al*[37]. Additionally, the increase in temperature may be correlated with a drier land as water is evaporated[38]. This may result in water stress in the plant of Bukit Cerakah thus, lower oxygen and hydrogen are supplied for the plant which is a necessity for its growth and activity as water is an important source of hydrogen and oxygen[39].

Furthermore, as the plant was placed under shade, this gives a colder condition in Sg. Buloh than in Bukit Cerakah. Cooler air provides higher relative humidity than warmer air[40] indicating that the humidity in Sg. Buloh is higher. So, the results of higher asiaticoside in plants of Sg. Buloh is in line with the finding from a study by Randriamampionona and friends[41] in 2007 that reported enhancement of asiaticoside in elevated humidity and also a study in 2012 by Rahajanirina *et al.,*[42] that concluded rainy season results in more asiaticoside content compared to the dry season.

When comparing the *C. asiatica* of Sg. Buloh and Sungkai side by side, are located in shades but the asiaticoside derived from the latter is far reduced. The reason underlying may be due to the plant being located at a hot spring (Sungkai) hence, hot flowing water varying from 50°C to 110°C as reported by Chia and colleagues[43] may affect the soil temperature and hence, the plant development as it is more strongly correlated with soil temperature than with air temperature[44]. Previous studies have reported that lower soil temperature may result in more fibrous and elongated roots which is better for water and nutrient uptake[44,45]. Furthermore, lower soil temperature was found to give higher plant secondary metabolites such as in the study on berries and tomatoes[44,46]. Therefore, the high soil temperature in Sungkai reduces the plant root development causing the uptake of water and nutrients to be impeded, retarding the plant growth and thereby, declining the biosynthesis of asiaticoside.

As depicted in Figure 2, the observed xylem of *C. asiatica* from Sg. Buloh was thicker compared to the other remaining regions. A thick xylem may indicate that the plant consumed optimum nutrients for its development such that boron is needed for the synthesis of lignin to strengthen the xylem[47]. Besides that, the xylem functions in water and nutrient transportation from the roots to the leaves[48]. A thicker xylem is important to provide a rigid structure to prevent them from rupturing due to the tension force and water pressure when delivering the water and nutrients[48]. The explanation behind the thicker xylem is consistent with the finding that the soil from Sg. Buloh is more acidic than the others as illustrated in Table 2.

As the availability of nutrients is influenced by the pH of the soil, more acidic soil is favourable in presenting most micronutrients needed for plant development compared to only slightly acidic soil such as in Bukit Cerakah and Sungkai. Thus, the thicker xylem in *pegaga* from Sg. Buloh is due to the rigid structure needed to be able to deliver nutrients and water throughout the plant whilst preventing it from collapsing[48], making it possible to transport materials for plant biological function and thereby, the plant's secondary metabolism can be conducted. Other than that, the higher asiaticoside content of *C. asiatica* from Sg. Buloh means that this plant is an acidic lover condition since it can carry out biosynthesis more efficiently. Moreover, since the plant is under nursery care, the plant soil might have been fertilised at a higher rate than in Bukit Cerakah and Sungkai. Thus, a higher fertiliser rate or concentration improves soil fertility as additional nutrients are available for the plant's usage and this may result in improved biosynthesis of terpenoids[10,49].

Another factor that possibly affects the synthesis of the plant's active constituent is the soil composition or characteristic. The type of plant-soil of Sg. Buloh, Bukit Cerakah and Sungkai are clayey soil, sandy soil and peaty humus soil respectively. The clayey soils have very tiny particles of less than 0.002mm in diameter in general, with very small pore spaces called micro pores[50]. Due to presence of numerous times more pore spaces, clay soils have greater total pore space than sandy soils[51]. Compared to macro pores, capillary action is substantially more pronounced in micropores. This makes the clay soils have superior soil water status than sandy soils because they absorb and hold significantly more water than sandy soils[50,51]. The optimum water and nutrient uptake allow the plant to work more efficiently, giving the highest secondary metabolite out of the plants from three different regions.

Contrasting the clayey soil (Sg. Buloh), the structure of the sandy soil is dry and friable[50]. Due to the large pore gaps (macro pores) and relatively large soil particle size, they frequently dry out quickly, making the soil low in water and nutrients as macro pores aid in water

infiltration and percolation, but they don't hold much water for utilisation by crops[51]. In addition, because the location of the plant is directly exposed to the sun as opposed to the other locations, which are semi-shaded, this results in more water loss as water[38] and fertiliser tend to leach out of the soil and escape to waterways before the plant can extract them, leading to water stress like have been explained earlier. When deprived of water, plant photosynthesis can be greatly restricted. Besides, this leads to nutrient uptake impairment since water plays an important role in the transportation of nutrients from soil to the roots[39] through the rate of water flow that controls the amount of nutrients such as nitrogen, magnesium and calcium being transported to the root surface before it is delivered throughout the plant[52].

As a result of the decomposing leaves, twigs, and other organic debris that have been littered on the bed of the hot spring, the peaty humus soil from Sungkai may be rich in organic matter[53]. However, this kind of peaty soil retains a lot of water, making drainage a must[50,53]. This poorly drained characteristic may cause waterlogging[54]. This may further lead to oxygen stress because the roots of the plant cannot receive enough oxygen, which can stunt or kill the plant as the spaces between soil particles contain air that provides oxygen[54]. Living cells, including root cells, need oxygen to break down sugars and release energy for plant survival and growth[55]. So, in the situation where the soil has excessive water in its pores and becomes saturated, oxygen deprivation may occur as oxygen is not delivered to the roots. This leads to the inability of the plant to carry out efficient root respiration and any other vital process[55], its growth is stunted and so the biosynthesis of a secondary metabolite which in this study, the asiaticoside of *C.* asiatica from Sungkai is much lesser than in *pegaga* of other locations.

In addition to the soil characteristics, its chemical properties such as the Cation Exchange Capacity (CEC) are also essential in providing the nutrients with mobility. When there is water in the soil, it helps in the transportation of nutrients from the soil into the roots[52]. The nutrients that are more readily leached are typically those that are being held by the soil particles less tightly. Higher CEC values improve the ability of soil particles to hold and retain nutrients hence, improving the soil fertility[52,53]. For example, because calcium (a divalent cation) is more tightly bound to the soil particles than potassium (a monovalent cation), potassium will leach more quickly and be transported to the roots[52]. Organic matter content in soil improves the CEC value[53]. This makes the soil of Sungkai the highest CEC value, followed by Sg. Buloh and then Bukit Cerakah. However, the asiaticoside content from *pegaga* of Sungkai is still the lowest despite having a higher CEC, higher nutrient retaining capability and improved soil fertility. This may be due to the above-discussed matters such as too high soil temperature and oxygen stress that disrupt the plant's vital processes and eventually decrease the asiaticoside content production.

5. Conclusion

To conclude, asiaticoside derived from *C. asiatica* of Sg.Buloh with a soil pH of 3.78 is the highest compared to the other two regions. The acidic condition of the soil in addition to clayey soil composition enhances the production of *C. asiatica* secondary metabolite (asiaticoside). Other environmental factors and growing conditions such as optimum soil water status, soil oxygen availability, soil fertility, temperature, light intensity and photoperiod, other than humidity may also improve the plant's secondary metabolism. Thus, these factors can be stimulated in future plant cultivation to improve crop yields and further enhance the biosynthesis of phytochemical components especially asiaticoside which holds numerous potential medicinal values so that the market demand can be fulfilled in addition to preventing the over-harvesting of the plant.

However, further research is required to investigate the differences in soil content (nutrients) that may affect the production of secondary metabolites. Other than that, factors such as light intensity often come hand in hand with temperature and water deprivation and thereby, there is a need to further determine the combined effects of multiple environmental factors that may induce the accumulation of bioactive compounds.

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7. Author Contributions

Nur Hazirah Zainal performed writing - original draft, review and editing, investigation and visualisation. Hannis Fadzilah Mohsin and Zolkapli Eshak performed validation and resources. Noor Anilizawatima Sulong performed writing - review and editing, conceptualization, methodology, validation, formal analysis, visualisation, resources, supervision and funding acquisition.

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