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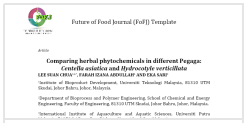
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Balas Teruskan

Article

Comparing herbal phytochemicals in different Pegaga: *Centella asiatica* and *Hydrocotyle verticillata*

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Abstract*:*

This study was aimed to reveal the differences of *Centella asiatica* and *Hydrocotyle verticillata.* Both species are known as Pegaga in local name and commonly eaten as salad in Malaysia. The phytochemical differences are important to prevent the misuse of the herbs in product development. The key phytochemical groups such as phenolics, flavonoids and terpenoids were estimated from the calorimetric assays, and subsequently identified the intense compounds using LC-MS/MS. The reported triterpenoids (asiatic acid and madecassic acid) and their trisaccharides (asiaticoside and madecassoside) were detected in *C. asiatica*. Glycosylated quercetin and rhamnocitrin were found in *H. verticillata*, but absent in *C. asiatica*. Quercetin and rutin appeared to be the compounds differentiating *H. verticillata* from *C. asiatica* based on unsupervised multivariate data analysis. The leaf images of the herbs were compared using a computational edge detection technique. The leaf morphology based on the leaf shape and vein pattern could clearly differentiate the herbs. Therefore, the application of the herbs in product formulation should be careful, since both herbs have different phytochemical profiles which would contribute to different biological activities.

Keywords: *Centella asiatica*, *Hydrocotyle verticillata*, pentacyclic triterpenoids, leaf morphology, LC-MS/MS.

1. Introduction

Centella asiatica (L.) Urban which is commonly known as Indian pennywort, Asiatic pennywort or gotu kola is a perennial herb belonging to the plant family Apiaceae

(formerly Umbelliferae). It was formerly named as *Hydrocotyle asiatica*, and then transferred to the genus of Centella by Ignatz Urban in 1879 (Urban, 1879). It can usually be found in the temperate and tropical swampy areas in Southeast Asian countries such as India, Sri Lanka, China, Indonesia, and Malaysia, as well as South Africa and Madagascar (Jamil, et al., 2007). This herb is one of the most commonly used herbs which has been claimed to possess various pharmacological effects, particularly on wound healing, maintenance of connective tissue, inhibition of excessive scar tissue (keloids) and treatment of various skin conditions such as ulcers, eczema and psoriasis (Brinkhaus, et al., 2000; Mangas, et al., 2008; Gohil, et al., 2010). The healing effects are mainly due to the presence of active constituents such as pentacyclic triterpenoids (asiatic acid and madecassic acid), and their trisaccharides (asiaticoside and madecassoside) (Nagoor Meeran, et al., 2018). These triterpenoid saponins and their sapogenins are also responsible for memory enhancement, haemostatic and venous hypertension (Gohil, et al., 2010; Chaisawang, et al., 2017; Nagoor, et al., 2018). Asiatic acid was proven to be effective against malignant glioma which is one of the most damaging and incurable tumors in brain (Kavitha, et al., 2011). The other phytochemicals include plant sterols, phenolics and flavonoids (Srivasta, et al., 1997).

This herb has been widely used as folk remedies for thousands of years (Diwan, et al., 1991). Recent publication also supports the beneficial use of the herb through scientific studies. Scientists and researchers are getting interested to generate technical data in line with the traditional remedies. The ever-increasing use of the herb has caused the problem of adulteration purposely or unintentionally with cheaper material. The common material that has been mistreated is *Hydrocotyle bonariensis* Comm. ex Lam, which is usually called as largeleaf pennywort or coast pennywort from the plant family Araliaceae (Plunkett, et al., 2004). This exotic aquatic macrophyte is also called as Ulam Pegaga which means Pegaga salad in Malaysia. Similar phenomenon is happening in Indonesia. The researchers reported that *C. asiatica* is potentially adulterated with either *Hydrocotyle verticillata* or *Merremia emarginata* which have same local name as Pegagan (Subositi, et al., 2016; Maruzy, et al., 2020). The misidentification has also been happened in Philippines by local folks (Daminar & Bajo, 2013). *H. bonariensis* is primarily planted in canals and water features for aesthetics and phytoremediation (Strosnider, et al., 2011). The juice of the plant is traditionally prepared to treat fever, colds, coughs, hepatities, infuenza, pruritus and sore throat, as well as headaches and urinary problems (Sujanapal & Sankaran, 2016). In 2014, a group of researchers from Singapore compared the vegetative differences of *C. asiatica* and *H. verticillata*. *H. verticillate* which is also known as water pennywort or whorled marsh-pennywort, is an exotic aquatic macrophyte that is commonly found in marshes. The difference between both species, in term of phytochemicals is extremely limited in literature. The difference of phytochemicals in both species is of great importance, especially for herbal product formulation.

Plant recognition is still the specialization of plant taxonomists and botanists with adequate experience to authenticate plant species. The advancement of computing

technologies and invention of digital cameras have supported the works of non-specialists. The approach is known as digital image processing which eases herbal identification in a rapid, simple, and effective manner. The leaf features such as edge or shape, vein, dimension and colour appear to be reliable inputs being considered in computing. Works have been extensively carried out on leaf image processing and plant classification using different algorithms (Azlah, et al., 2019). To the best of our knowledge, studies have not been performed to relate phytochemicals and leaf morphological observation for plant recognition. Most probably, there are two different fields of studies in which cross disciplinary collaboration is relatively limited in academia. Therefore, this study was carried out to investigate the differences of phytochemicals and leaf morphology between *C. asiatica* and *H. verticillata* which are commonly mistreated for product formulation in the market.

2. Materials and Methods

2.1. Phytochemical extraction

Phytochemical extraction was conducted using 1 g powdered leaves and stems in 100 mL solvent systems consisted of different concentrations of ethanol ranged from 0-100 %v/v. The mixture was refluxed at the boiling points of the solvent systems for 2 hours. The supernatant was collected after centrifuged and filtered by Whatman cellulose filter paper (Grade 1, 110 mm x 11 µm). The supernatant was then concentrated using a rotary evaporator and dried in an oven at 50 ^{o}C until dryness. The weight of dried crude extract was recorded. All experiments were carried out in triplicate, unless otherwise stated.

2.2. Total Phenolic Content

The total phenolic content of samples was estimated using the colorimetric method according to the procedures described by Siddiqui et al. (2017) with modification. Different concentrations of samples were reconstituted in 50% methanol. About 1 mL methanolic sample was mixed with 5 mL Folin–Ciocalteu reagent which were previously diluted with deionized water. The mixture was left for 5 min at 25 ^{o}C and then added with 5 mL sodium carbonate (7.5%). After incubation for 20 min, the absorbance of the mixture was measured using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) at 760 nm. A calibration curve of standard chemical, gallic acid (0 - 100 µg/mL) was constructed and the results are expressed as milligram gallic acid equivalent per gram sample (mg GAE/g).

2.3. Total Flavonoid Content

The total flavonoid content of samples was also estimated using the colorimetric method (Aryal, et al., 2019). An aliquot of 1 mL sample was mixed with 3 mL methanolic $AlCl_3$ solution (10 %w/v), 0.2 mL potassium acetate (1 M) and 5.6 mL distilled water.

The mixture was incubated at 25 °C for 30 min and followed by the measurement of absorbance at 420 nm using a UV-Vis spectrophotometer. The results are expressed as milligram quercetin equivalent per gram sample (mg QE/g).

2.4. Total triterpenoid content

The total triterpenoid content was estimated spectrophotometrically using vanillin assay (Chua, et al., 2019). The 1 mg/mL methanolic sample (250 µL) was added into a test tube containing 8g/100 mL vanillin (250 µL) and topped up with 72 % sulfuric acid (2.5 mL). The mixture of the solution was heated for 10 min at 60 °C, and subsequently cooled in an ice-water bath for 5 min. The absorbance of the solution was recorded by a UV-vis spectrophotometer at 544 nm. Diosgenin (5.7–71.4 mg/L) was used as the standard chemical to build a calibration curve. The results are expressed as mg diosgenin equivalent per g sample (mg DE/g).

2.5. Free radical scavenging activity

The antiradical capacity of samples was determined using DPPH (2,2-diphenyl-2-picrylhydrazyl) assay as described by Chu et al. (2000). A 2 mL sample at different concentrations ranged from 100-500 µg/mL was added into 2 mL methanolic DPPH (0.1 mM) solution. The mixture was kept aside in a dark area for 30 min. The absorbance of the solution was measured at 517 nm spectrophotometrically. BHA was used as the standard chemical for a calibration curve construction. The percentage of radical inhibition was calculated using Equation 1. The results are expressed as effective concentration at 50% inhibition (IC50).

$$\text{Inhibition (\%)} = \frac{A_o - A_S}{A_o} \; x \; 100 \quad (1)$$

Where A_o = absorbance of control and A_S = absorbance of sample.

2.6. Cation radical scavenging activity

The cation radical inhibition of sample was determined using ABTS (2,2'azinobis(3-ethylbenzothiozoline-6-sulfonic acid) disodium salt) assay according to the method described by Biskup et al. (2013) with some modifications. The $ABTS^{\bullet+}$ solution was prepared by reacting ABTS (7 mM) with potassium persulfate (2.45 mM) at a ratio of 1:1, and incubated overnight in a dark place. The solution was then diluted with 50% methanol to have an absorbance of 1.00 at 734 nm. Samples were also dissolved in 50% methanol in the concentration of 0 to 1,000 mg/mL. Then, 2 mL of the diluted $ABTS^{\bullet+}$ was added with 100 µL sample solution, and incubated for 6 min under subdued light condition. The absorbance was measured at 734 nm using a UV-Vis spectrophotometer.

2.7. Reducing power

The reducing power of samples was determined using ferric reducing antioxidant power (FRAP) assay which was carried out according to the procedures reported by Chua et al. (2013) with modification. FRAP reagent was freshly prepared by mixing 2.5

mL 2,4,6-tripydyl-s-triazine complex (10 mM, Fe^{3+}-TPTZ) in hydrochloric acid (40 mM), 2.5 mL iron (III) chloride (20 mM, $FeCl_3$) and 25 mL acetate buffer (0.3 M, pH 3.6). The reagent solution was kept in the dark at 37°C before use. Sample (0.2 mL) was mixed with 1.8 mL FRAP reagent, and incubated at room temperature under subdued light condition for 10 min. The absorbance was measured at 593 nm using a UV-Vis spectrophotometer. Ascorbic acid (10 mg/L) was used as standard chemical.

2.8. Compound screening by LC-MS/MS

A Liquid chromatography (Ultimate 3000; Dionex Corporation; Sunnyvale, CA, USA) integrated with a diode array detector (Dionex Ultimate 3000) and a tandem mass spectrometer (QSTAR Elite; AB Sciex; Foster City, CA, USA) was used for compound screening. Compounds were separated by a C18 XSelect HSS T3 column (2.1 mm × 100 mm, 2.5 µm) at a flow rate of 150 µL/min. A binary solvent system consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile) was used as the mobile phase at the following gradient: 0–10 min, 10% B; 10–20 min, 10–80% B; 20–25 min, 80% B; 25–25.1 min, 80–10% B; and 25.1–30 min, 10% B. The injection volume was 5 µL. Compounds were eluted from the column and detected at the wavelength of 254 nm. Subsequently, compounds were ionized by a turbo ion spray (-4,500 V) before mass detection at the negative ion mode. The mass range was set at the range of 100–1000 m/z. Nitrogen gas was used for curtain gas (25 psi) and nebulizing gas (40 psi). The declustering potential was 40 V, whereas the focusing potential was 200 V. Samples were filtered using a 0.2 µm nylon membrane filter prior to injection.

2.9. Leaf morphological recognition

An in-house leaf image recognition system which was developed using the Java programming language was used to process the leaf images of both herbal species, namely *C. asiatica* and *H. verticillata*. The leaf image of each plant species was uploaded into the system for image processing and feature extraction. The leaf images were pre-processed via segmentation, grayscale conversion and noise removal. The key features such as leaf edge, vein pattern and dimension were extracted from the processed images using a serial of algorithms. Prewitt and thinning algorithms were used for edge detection. The algorithms of CheckLines, CheckLineLength, paintLines and paintPoints were used to construct the vein pattern of leaves. An array of tokens was designed to identify the coordinates of lines using cosinus and sinus angles for the determination of diagonal dimension.

2.10. Multivariate data analysis

An unsupervised principal component analysis was carried out using a Pareto scaling in the data processing software (MarkerView 1.2.1, Applied Biosystems/MDSSciex, Foster City, CA, USA). The parameters for peak finding and alignment were set as minimum peak width, 0.05 Da; mass tolerance, 0.01 Da and retention time tolerance, 0.5 min.

3. Results and discussion

3.1. High throughput mass screening

A high throughput mass screening was performed to detect phytochemicals in *C. asiatica* extracts which were prepared using different concentrations of ethanol ranged from 0-100%. The previously reported phytochemicals such as phenolic acids (caffeoylquinic acid, dicaffeoylquinic acid and dicaffeoyl methoxyoxaloylquinic acid), flavonoids (kaempferol, quercetin and glucuronyl quercetin) and triterpenoids (asiatic acid, madecassic acid, asiaticoside and madecasspside) were detected in this study. The intensities of the compound peaks are plotted in Figure 1. The figure shows that madecassic acid has the highest peak intensity, and followed by asiatic acid among the detected phytochemicals. The figure also shows that 50% ethanol is likely to be the most effective ethanol composition in the solvent system for the phytochemicals extraction.

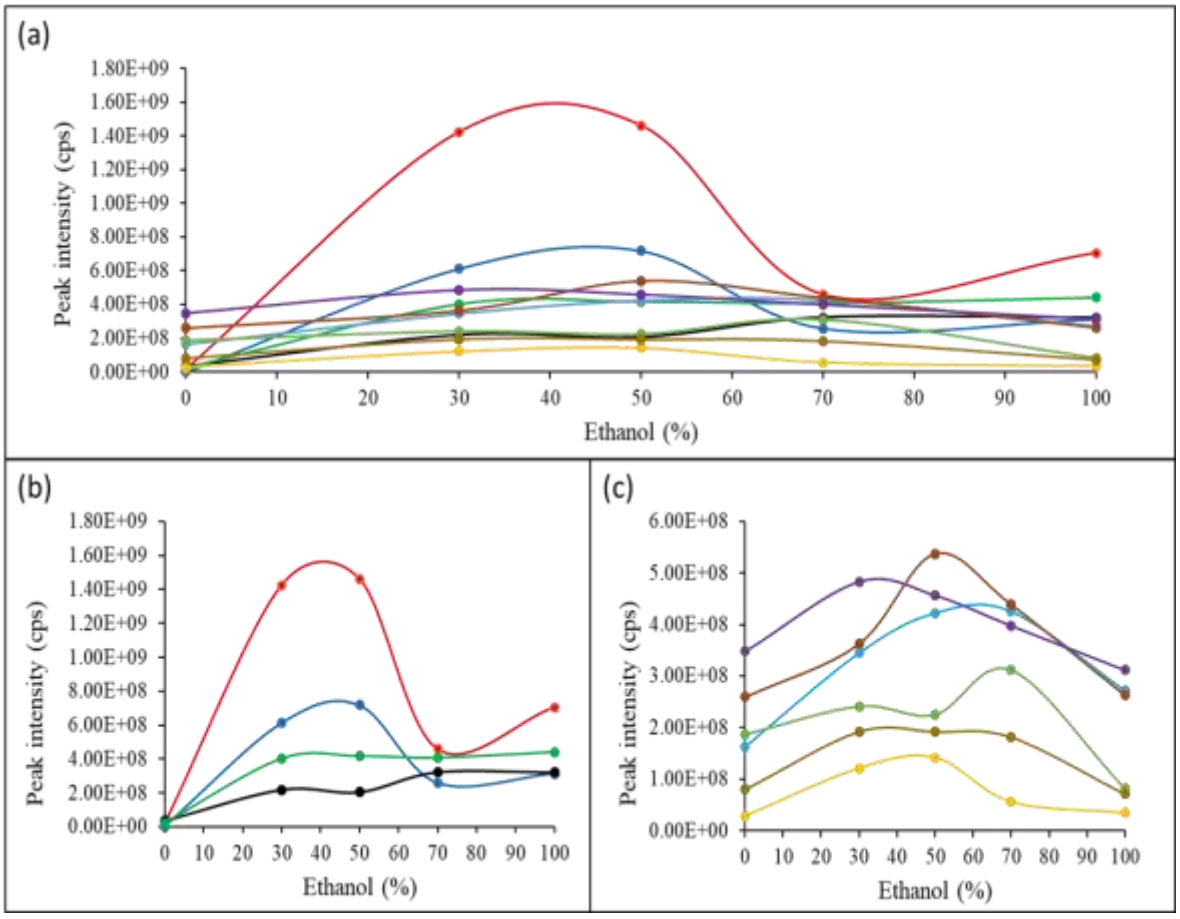

Figure 1. (a) Ten target phytochemicals consisted of (b) two triterpenoids and their trisaccharides and (c) three phenolic acids, two flavonoids and one glycosylated flavonoid in the extracts of *Centella asiatica* prepared using different ethanol concentrations, where asiatic acid, madecassic acid, asiaticoside, madecassoside, caffeoylquinic acid,

dicaffeoylquinic acid, dicaffeoylmethoxyoxaloylquinic acid, quercetin, kaempferol and glucuronyl quercetin

In the subsequent analysis, 50% ethanolic extracts of the leaves and pericladial petioles of *C. asiatica* were examined for total phenolic, flavonoid and triterpenoid content spectrophotometrically (Figure 2). The results showed that leaf extract exhibited higher content of phytochemicals such as phenolics, flavonoids and triterpenoids than pericladial petiole extract. The proximate content of phytochemicals was also compared with its mimicking counterpart, *H. verticillata*. The comparison revealed that both herbal species had different compositions of phytochemicals, and phenolics was being the largest phytochemical group in the samples (Figure 2).

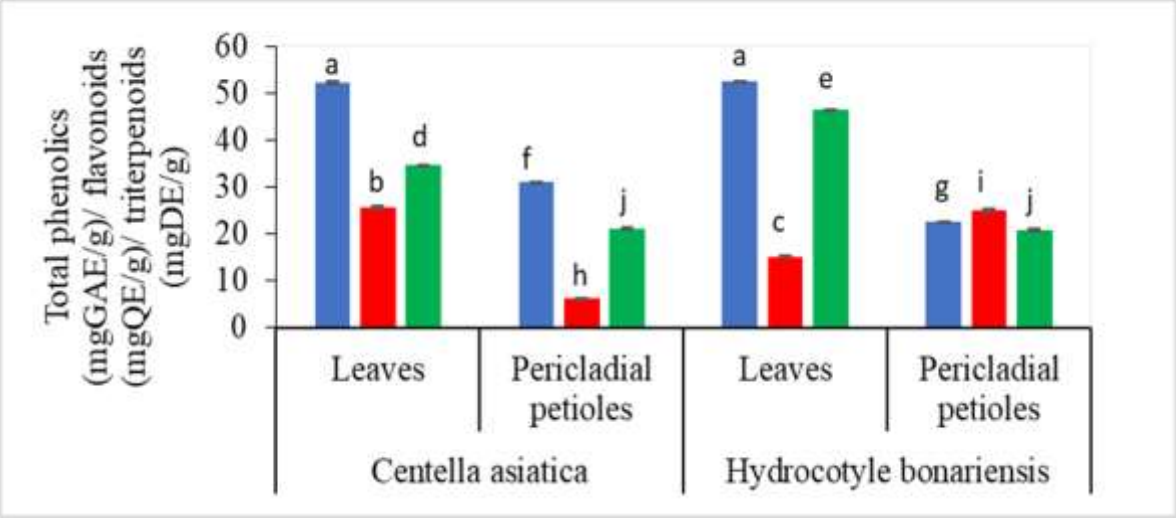

Figure 2. Total phenolics (blue bar), flavonoids (red bar) and triterpenoids (green bar) of the leaf and pericladial petiole extracts from *Centella asiatica* and *Hydrocotyle verticillata*. One-way analysis of variance (ANOVA) followed by T-test paired two samples for means were conducted to determine the significant difference of phytochemical content in the leaf samples of *C. asiatica* and *H. verticillate*, and in the pericladial petiole samples of *C. asiatica* and *H. verticillate*. Different small letters indicate the significant difference at $p < 0.05$.

Total phenolic content was determined using the widely accepted Folin-Ciocalteu assay. This assay is a non-specific phenol oxidation in alkaline medium catalyzed by two strong inorganic oxidants, namely phosphotungstic and phosphomolibdic acids. The heteropoly acid was reduced from the valence state of +6 to +5, and resulting the formation of blue molybdenum-tungsten complex for absorbance measurement. The other non-phenolic organic and inorganic compounds could possibly contribute to an elevated apparent phenolic content. Hence, the assay actually describes the total reducing capacity of a sample which is often correlated to its antioxidant activity.

In the present study, quercetin was used as a standard chemical to build the calibration curve of total flavonoid content. The absorbance was attributed to the formation of acid labile complexes after chelating flavonoids with aluminum ions. Possibly, the C-4 keto, C-3 or C-5 hydroxyl groups and ortho-dihydroxyl groups in the A or B rings of flavonoids may chelate with aluminum ions to produce colored complex for detection (Kasprzak, et al., 2015). The use of aluminum ions in the presence of acetate salt was more suitable for flavonols (Pekal & Pyrzynska, 2014).

The antioxidant capacity of the herbal extracts was also evaluated in terms of scavenging free and cation radicals, as well as reducing ferric ions as presented in Figure 3. In line with the proximate content of phytochemicals, the antioxidant capacity of leaf extract was higher than its pericladial petiole extract. This is because the antioxidant capacity of plant extract is mostly attributed to the presence of phytochemicals, particularly phenolic acids and flavonoids. The figure also clearly shows that the leaf extract of *C. asiatica* could exhibit the highest scavenging activities against free and cation radicals, and reducing power. The 50% ethanolic extract was also found to be an effective radical scavenger compared to its capacity as a reducing agent. This was because the concentration of extract which was required to inhibit 50% of radicals was lower than that value to reduce ferric ions. The scavenging activity could achieve more than 80%, whereas the reducing power was about 70% which was about 10% lower than its scavenging capacity.

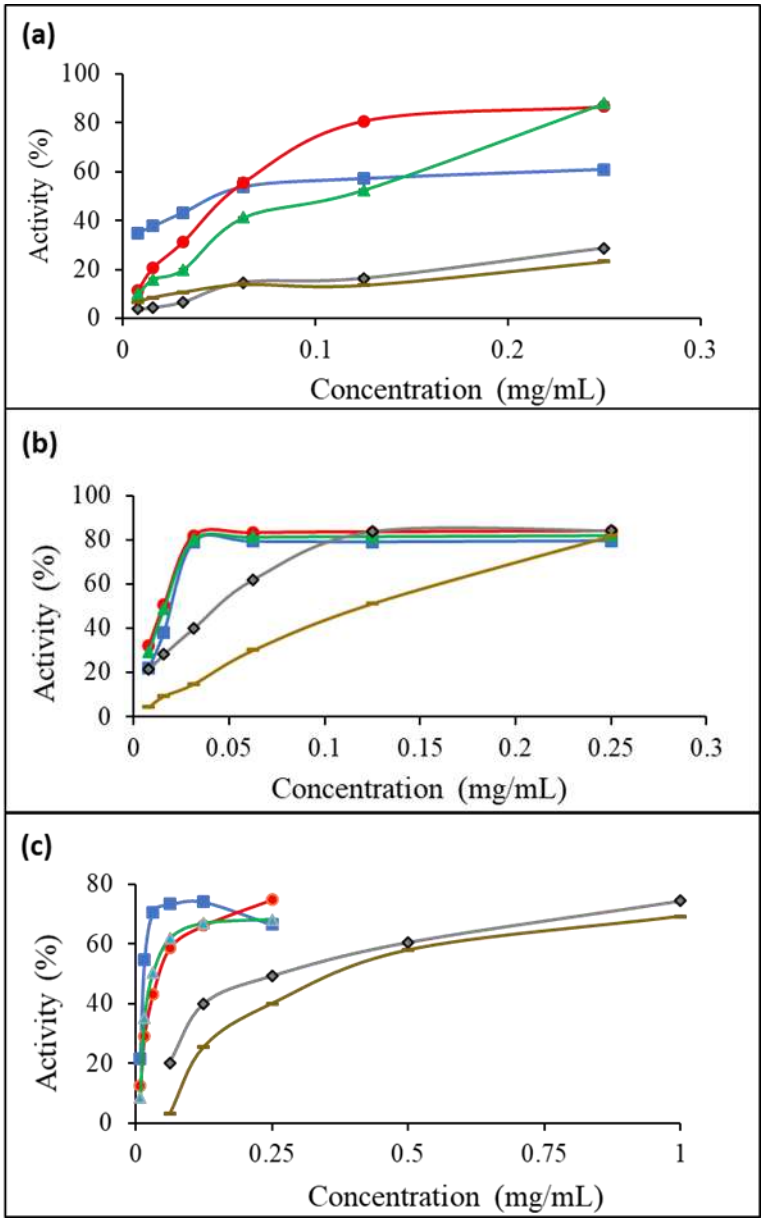

Figure 3. Antioxidant capacity of extracts based on the scavenging activities of (a) free radicals and (b) cation radicals, as well as (c) reducing power of ferric ions for (■) ascorbic acid, (●) the leaf extract of *Centella asiatica*, (▲) the leaf extract of *Hydrocotyle verticillate*, (♦) the pericladial petiole of *C. asiatica* and (−) the pericladial petiole of *H. verticillate*.

The antioxidant compounds primarily follow the electron transfer mechanism to inhibit the radicals. The compounds might also involve in hydrogen atom transfer at a slower rate (Gulcin, 2020). Therefore, compounds with bulky rings would have the difficulty to access radicals for electron transfer. On the other hand, compounds with conjugated

double bonds and multiple hydroxyl groups would be the dominant chemical characteristics to inhibit radicals. DPPH assay is considered to be more selective because aromatic acid with a single hydroxyl group does not react with DPPH radicals (Cerretani & Bendini, 2010). This also indicates that the leaf extract of *C. asiatica* may have many polyol phenolics either from the group of phenolic acids or polyphenols.

Compounds react with ABTS radicals would also respond to the FRAP assay because of similarity in redox potentials (Gulcin, 2020). However, the results showed to have higher concentration of samples to inhibit 50% of ferric ions. The lower reducing power could only be contributed by water soluble antioxidative compounds (Apak, et al., 2007). The acidic medium of FRAP assay was used to promote ferric ion solubility which indirectly increased the redox potential. Pulido et al. (2000) reported that the absorbance of compounds such as caffeic acid, quercetin and tannic acid was not stabilized even after several hours of reaction time in FRAP assay. The observation was in good agreement with previous researchers that antioxidant activity measured in FRAP assay was lower than that in ABTS assay (Gulcin, 2020).

The variance of phytochemicals in both herbs could be clustered into 3 major principal components. The unsupervised multivariate analysis indicated that the phytochemicals in both herbs could achieve up to 78.4 % of the total variance for the first principal component (PC1). Figure 4 shows the phytochemicals in *C. asiatica* are prone to be located at the positive region, whereas the phytochemicals in *H. hydrocotyle* are mostly located at the negative region of PC1. The phytochemicals such as m/z 301 (quercetin), 353 (caffeoylquinic acid), 609 (glucosylrhamnosyl quercetin or rutin), 721 (tricaffeoyl-2,7-anhydro-2-octulopyranosonic acid) and 1101 (saponin) are likely to be the dominant compounds differentiating *H. hydrocotyle* from *C. asiatica* (Figure 4(c)). Although m/z 461 (unknown), 477 (glucuronyl quercetin), 515 (glycosyl caffeoylquinate) and 601 (dicaffeoyl methoxyoxaloylquinic acid) were found in both plant species, they were present in higher amount in *C. asiatica* (Figure 4(d)). The pentacyclic triterpenoids and their trisaccharides were located near the center of the axis as indicated in Figure 4.

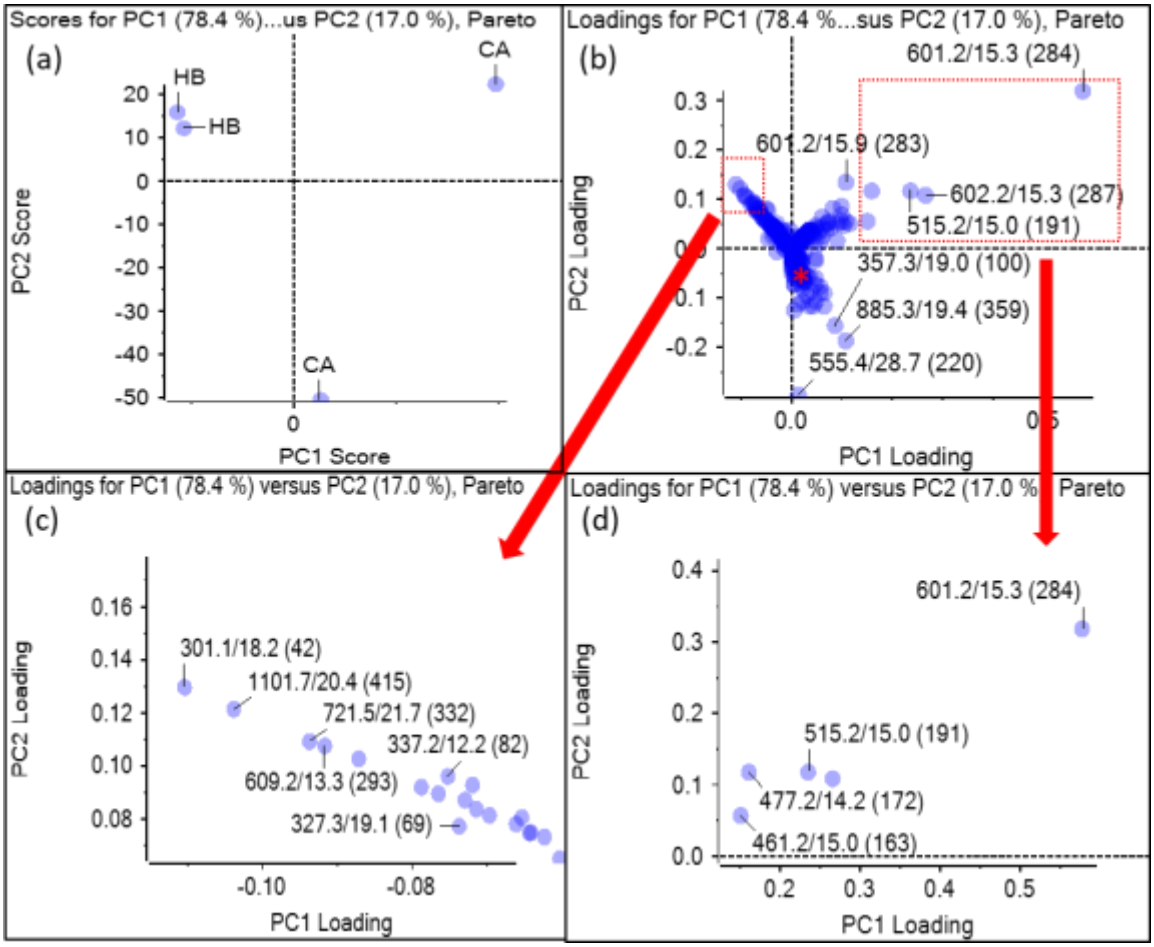

Figure 4. (a) Score and (b) loading plots of *Centella asiatica* (CA) and *Hydrocotyle verticillata* (HB) with the zoom-in area of masses, specifically for (c) HB in the negative region and (d) CA in the positive region of first principal component. * is the location of the pentacyclic triterpenoids and their trisaccharides in *C. asiatica*.

3.2. Comparison of target phytochemicals

The presence of selected phytochemicals was then compared in both 50% ethanolic extracts of *C. asiatica* and *H. verticillata*. The comparison is made in term of its peak intensity as presented in Figure 5 (supplementary). The figure clearly illustrates that *C. asiatica* has higher content of the target phytochemicals, except for caffeoylquinic acid and quercetin. This could support the belief that *C. asiatica* is more active for ethnomedicine, especially for gastrointestinal disorders like dysentery, constipation, stomach problems, indigestion and loss of appetite, and for memory enhancement (Jahan, et al., 2012). Interestingly, there were a few of glycosylated polyphenols detected only in the extract of *H. verticillata* as listed in Table 1 (Supplementary). The quick mass screening results indicated that *C. asiatica* had higher triterpenoids and their glycosides, whereas *H. verticillata* contained more polyphenols and their

glycosides. Previous researchers from Taiwan also reported the detection of quercetin, isorhamnetin and rutin in Hydrocotyle species (Huang, et al., 2008; Yang, et al., 2008). The results revealed that both species are totally different in phytochemical profile, even they are locally called as Pegaga. The difference in phytochemical profile most possibly will contribute to pharmacological variance.

3.3. Differentiation of leaf morphology

The leaf images of both plant species were also processed using the established computing system for comparison. This is one of the non-destructive and rapid recognition techniques for plant recognition. The leaf edge including shape, vein pattern and dimension are selected as the dominant leaf features for the differentiation of plant species (Ehsani Rad, 2010; Lee & Hong, 2013). The leaf colour was not considered because this feature might be changed due to the seasonal and environmental factors.

The edge of plant leaves is the most obvious and easily recognised feature for identification. Prewitt algorithm was used to detect the edge of leaves in this study. This algorithm has been proven for its reliability for the leaf classification and plant disease detection in previous studies. (Navarajan, et al., 2015; Vilasini Ramamoorthy, 2020). The detected edge points produced pixels forming the leaf edge and vein as presented in Figure 6 (supplementary). From the pixels produced by Prewitt algorithm, it is clearly indicated that both species of plants have different shapes and vein patterns morphologically. The leaves of *C. asiatica* show to have kidney shape with second order veins branched off at the intervals of several first order veins, and reticulate meshes could also be observed between the third order veins and minor veins. On the other hand, the round shaped leaf of *H. verticillata* displays multiple first order veins.

Vein pattern could be the fingerprint of plants which is sometimes not easily observed without the assistance of pattern recognition tool (Scoffoni, et al., 2008). Therefore, the use of high performance computing system would be the method of choice. Besides phytochemical identification, leaf morphology including the vein pattern has been recognized as a reliable tool in identifying plant species. In the present study, both *C. asiatica* and *H. verticillata* belong to palmately veined species with multiple first order veins branching from the petiole (Sack, et al., 2008). The venation architecture is important to determine the sensitivity hydraulic conductance of leaves. A clear correlation has been established between the vein characteristics and properties of leaves, particularly on the aspects of leaf damage and drought tolerance (Scoffoni, et al., 2011; Sack, et al., 2008).

5. Conclusions

It is important to highlight that the difference of phytochemicals in *C. asiatica* and *H. verticillata*, even though both species are known as Pegaga in Malaysia. The findings of the study proved that *C. asiatica* contained pentacyclic triterpenoids (asiatic acid and madecassic acid) and their trisaccharides (asiaticoside and madecassocide), whereas *H. verticillata* contained high amount of quercetin and its glycosylated derivatives. The different venation of the plant leaves has also explained the variance of phytochemical profiles which would contribute to different biological activities.

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Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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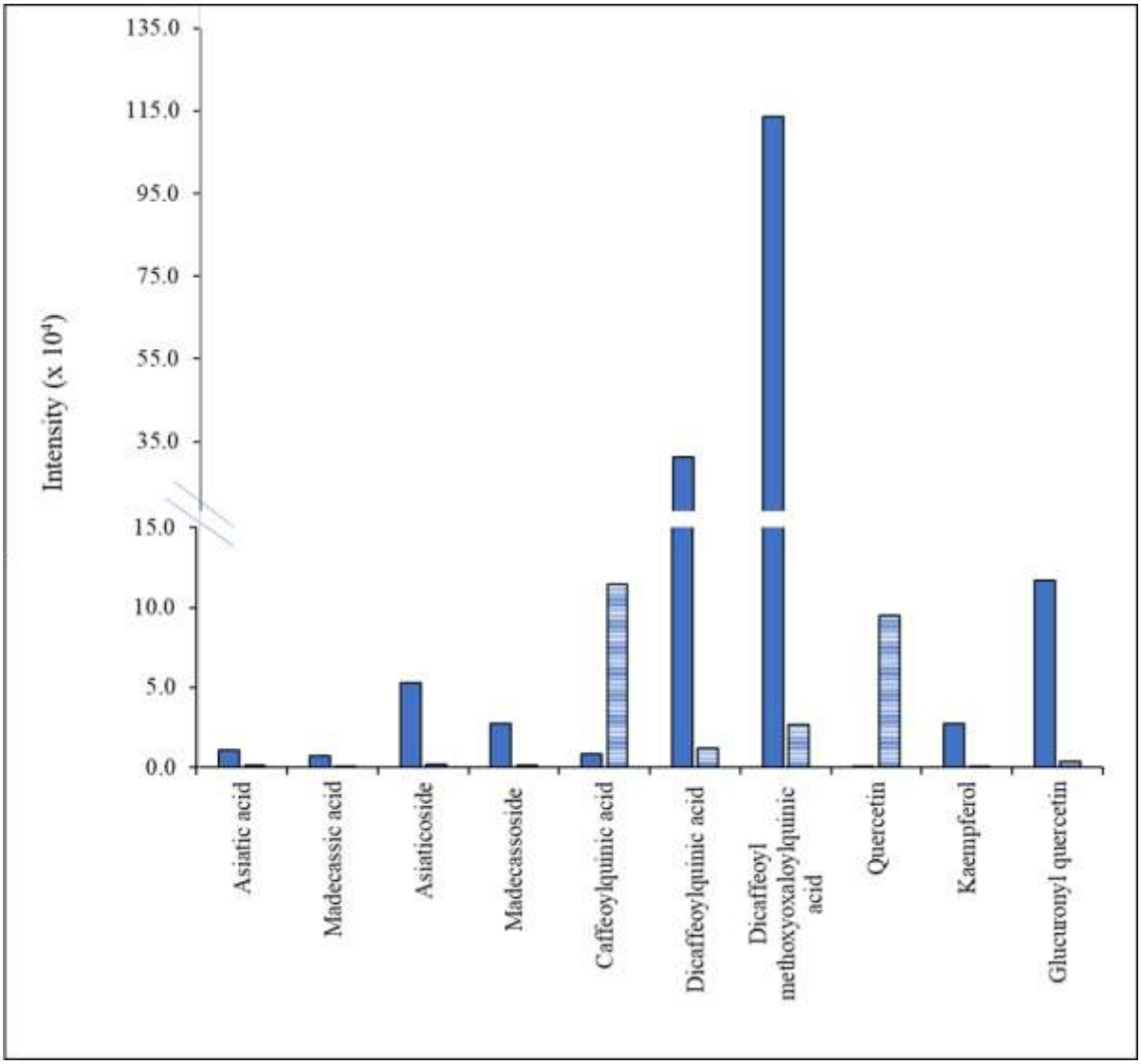
505 Supplementary



506

507 Figure 5 (supplementary). Target phytochemicals detected in *Centella asiatica* (solid
508 blue bar) and *Hydrocotyle verticillata* (line blue bar) extracts prepared using 50%
509 ethanol.

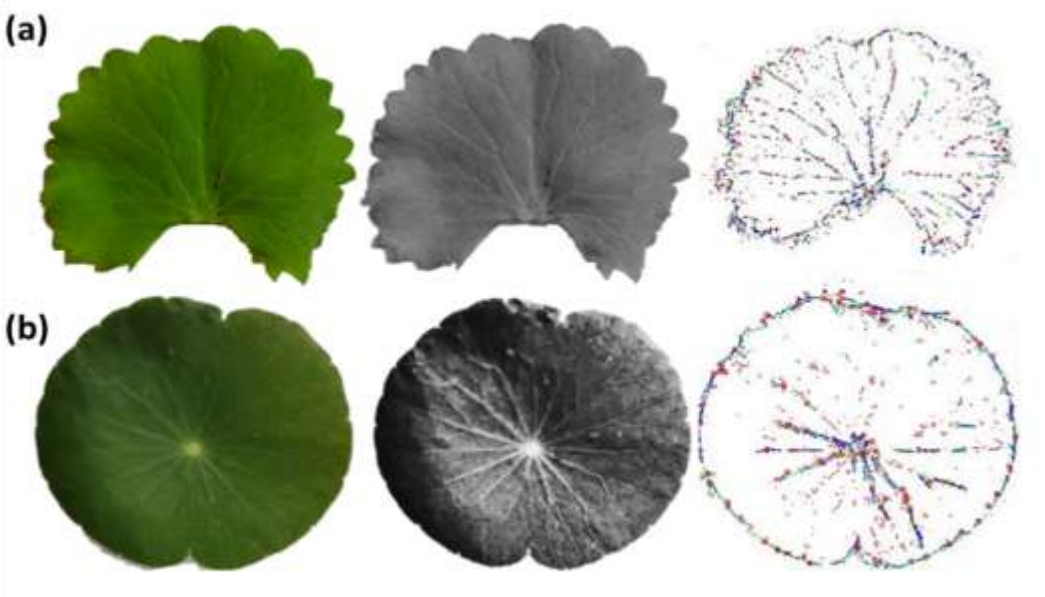


Figure 6 (supplementary). Original leaf morphological images of (a) *Centella asiatica* and (b) *Hydrocotyle verticillata* which are converted to gray scale and pixel images

Table 1 (supplementary). Phytochemicals detected in *Hydrocotyle verticillata* extract

Hydrocotyle verticillata	**Putative compounds**	**References**
301/273/179/151	quercetin	(Maulidiani, et al., 2014)
433/300(-133)/299/271	quercetin pentoside	(Maulidiani, et al., 2014)
447/300(-147)/283/271(-176)/255	isorhamnetin pentoside	(Li, et al., 2016)
463/300(-163)/271(-192)	quercertin glucoside	(Li, et al., 2016)
593/564/531(-62)/491/449/429/284/283(-310)/255/227	luteolin rutinoside	(Brito, et al., 2014)
609/507/361/300/271	rutin	(Maulidiani, et al., 2014)
639/463(-176)/300(-163)/269/255	caffeoyl rhamnocitrin glucuronide	(Chen, et al., 2016)
653/299(-354)/284	caffeoylquinoyl rhamnocitrin	(Chen, et al., 2016)
669/463(-206)/300(-369)/271(-398)/255	feruyl rhamnocitrin glucuronide	(Chen, et al., 2016)
695/300/299(-396)	rhamnocitrin tripentosides	(Chen, et al., 2016)

755/299bp(-456)/271(-484)	rhamnocitrin diglysoypentoside	(Chen, et al., 2016)
1187/581/285(-296)	$[2M-H]^-$, luteolin rutinoside	(Brito, et al., 2014)

515

Comparing ~~Reveal the~~ herbal phytochemicals [DC1][CL2] in~~of~~ different Pegaga [DC3][CL4]: *Centella asiatica* and *Hydrocotyle verticillata*

Abstract

This study was aimed to reveal the differences of *Centella asiatica* and *Hydrocotyle verticillata.* Both species are known as Pegaga in local name and commonly eaten as salad in Malaysia. The phytochemical differences are important to prevent the misuse of the herbs in product development. The key phytochemical groups such as phenolics, flavonoids and terpenoids were estimated from the calorimetric assays, and subsequently identified the intense compounds using LC-MS/MS. The reported triterpenoids (asiatic acid and madecassic acid) and their trisaccharides (asiaticoside and madecassoside) were detected in *C. asiatica*. Glycosylated quercetin and rhamnocitrin were found in *H. verticillata*, but absent in *C. asiatica*. Quercetin and rutin appeared to be the compounds differentiating *H. verticillata* from *C. asiatica* based on unsupervised multivariate data analysis. The leaf images of the herbs were compared using a computational edge detection technique. The leaf morphology based on the leaf shape and vein pattern could clearly differentiate the herbs. Therefore, the application of the herbs in product formulation should be careful, since both herbs have different phytochemical profiles which would contribute to different biological activities.

Keywords: *Centella asiatica*, *Hydrocotyle verticillata*, pentacyclic triterpenoids, leaf morphology, LC-MS/MS

1.

~~1.~~ Introduction

Centella asiatica (L.) Urban which is commonly known as Indian pennywort, Asiatic pennywort or gotu kola is a perennial herb belonging to the plant family Apiaceae (formerly Umbelliferae). It was formerly named as *Hydrocotyle asiatica*, and then transferred to the genus of Centella by Ignatz Urban in 1879 (Urban, 1879). It can usually be found in the temperate and tropical swampy areas in Southeast Asian countries such as India, Sri Lanka, China, Indonesia, and Malaysia, as well as South Africa and Madagascar (Jamil, et al., 2007). This herb is one of the most commonly used herbs which has been claimed to possess various pharmacological effects, particularly on wound healing, maintenance of connective tissue,

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inhibition of excessive scar tissue (keloids) and treatment of various skin conditions such as ulcers, eczema and psoriasis (Brinkhaus, et al., 2000; Mangas, et al., 2008; Gohil, et al., 2010). The healing effects are mainly due to the presence of active constituents such as pentacyclic triterpenoids (asiatic acid and madecassic acid), and their trisaccharides (asiaticoside and madecassoside) (Nagoor Meeran, et al., 2018). These triterpenoid saponins and their sapogenins are also responsible for memory enhancement, haemostatic and venous hypertension (Gohil, et al., 2010; Chaisawang, et al., 2017; Nagoor, et al., 2018). Asiatic acid was proven to be effective against malignant glioma which is one of the most damaging and incurable tumors in brain (Kavitha, et al., 2011). The other phytochemicals include plant sterols, phenolics and flavonoids (Srivasta, et al., 1997).

This herb has been widely used as folk remedies for thousands of years (Diwan, et al., 1991). Recent publication also supports the beneficial use of the herb through scientific studies. Scientists and researchers are getting interested to generate technical data in line with the traditional remedies. The ever-increasing use of the herb has caused the problem of adulteration purposely or unintentionally with cheaper material. The common material that has been mistreated is *Hydrocotyle bonariensis* Comm. ex Lam, which is usually called as largeleaf pennywort or coast pennywort from the plant family Araliaceae (Plunkett, et al., 2004). This exotic aquatic macrophyte is also called as Ulam Pegaga which means Pegaga salad in Malaysia. Similar phenomenon is happening in Indonesia. The researchers reported that *C. asiatica* is potentially adulterated with either *Hydrocotyle verticillata* or *Merremia emarginata* which have same local name as Pegagan (Subositi, et al., 2016; Maruzy, et al., 2020). The misidentification has also been happened in Philippines by local folks (Daminar & Bajo, 2013). *H. bonariensis* is primarily planted in canals and water features for aesthetics and phytoremediation (Strosnider, et al., 2011). The juice of the plant is traditionally prepared to treat fever, colds, coughs, hepatities, infuenza, pruritus and sore throat, as well as headaches and urinary problems (Sujanapal & Sankaran, 2016~~http://florawww.eeb.uconn.edu/ipm.html~~). [DC5][CL6] In 2014, a group of researchers from Singapore compared the vegetative differences of *C. asiatica* and *H. verticillata*. *H. verticillate* which is also known as water pennywort or whorled marsh-pennywort, is an exotic aquatic macrophyte that is commonly found in marshes. The difference between both species, in term of phytochemicals is extremely limited in literature. The difference of phytochemicals in both species is of great importance, especially for herbal product formulation.

Plant recognition is still the specialization of plant taxonomists and botanists with adequate experience to authenticate plant species. The advancement of computing technologies and invention of digital cameras have supported the works of non-specialists. The approach is known as digital image processing which eases herbal identification in a rapid, simple, and effective manner. The leaf features such as edge or shape, vein, dimension and colour appear to be reliable inputs being considered in computing. Works have been extensively carried out on leaf image processing and plant classification using different algorithms (Azlah, et al., 2019). To the best of our knowledge, studies have not been performed to relate phytochemicals and leaf morphological observation for plant recognition [DC7][CL8]. Most probably, there are two different fields of studies in which cross disciplinary collaboration is relatively limited in academia. Therefore, this study was carried out to investigate the

differences of phytochemicals and leaf morphology between *C. asiatica* and *H. verticillata* which are commonly mistreated for product formulation in the market.

~~2.~~ 2. Materials and Methods

2.1. —Phytochemical extraction

Phytochemical extraction was conducted using 1 g powdered leaves and stems in 100 mL solvent systems consisted of different concentrations of ethanol ranged from 0-100 %v/v. The mixture was refluxed at the boiling points of the solvent systems for 2 hours. The supernatant was collected after centrifuged and filtered by Whatman cellulose filter paper (Grade 1, 110 mm x 11 µm). The supernatant was then concentrated using a rotary evaporator and dried in an oven at 50 °C until dryness. The weight of dried crude extract was recorded. All experiments were carried out in triplicate, unless otherwise stated.[DC9][CL10]

2.2. —Total Phenolic Content

The total phenolic content of samples was estimated using the colorimetric method according to the procedures described by Siddiqui et al. (2017)[DC11][CL12] with modification. Different concentrations of samples were reconstituted in 50% methanol. About 1 mL methanolic sample was mixed with 5 mL Folin–Ciocalteu reagent which were previously diluted with deionized water. The mixture was left for 5 min at 25 °C and then added with 5 mL sodium carbonate (7.5%). After incubation for 20 min, the absorbance of the mixture was measured using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) at 760 nm. A calibration curve of standard chemical, gallic acid (0 - 100 µg/mL) was constructed and the results are expressed as milligram gallic acid equivalent per gram sample (mg GAE/g).

2.3. —Total Flavonoid Content

The total flavonoid content of samples was also estimated using the colorimetric method (Aryal, et al., 2019). An aliquot of 1 mL sample was mixed with 3 mL methanolic $AlCl_3$ solution (10 %w/v), 0.2 mL potassium acetate (1 M) and 5.6 mL distilled water. The mixture was incubated at 25 °C for 30 min and followed by the measurement of absorbance at 420 nm using a UV-Vis spectrophotometer. The results are expressed as milligram quercetin equivalent per gram sample (mg QE/g).

2.4. —Total triterpenoid content

The total triterpenoid content was estimated spectrophotometrically using vanillin assay (Chua, et al., 2019). The 1 mg/mL methanolic sample (250 µL) was added into a test tube containing 8g/100 mL vanillin (250 µL) and topped up with 72 % sulfuric acid (2.5 mL). The mixture of the solution was heated for 10 min at 60 °C, and subsequently cooled in an ice-water bath for 5 min. The absorbance of the solution was recorded by a UV-vis spectrophotometer at 544 nm. Diosgenin (5.7–71.4 mg/L) was used as the standard chemical to build a calibration curve. The results are expressed as mg diosgenin equivalent per g sample (mg DE/g).

2.5. —Free radical scavenging activity

The antiradical capacity of samples was determined using DPPH (2,2-diphenyl-2-picrylhydrazyl) assay as described by Chu et al. (2000). A 2 mL sample at different concentrations ranged from 100-500 µg/mL was added into 2 mL methanolic DPPH (0.1 mM) solution. The mixture was kept aside in a dark area for 30 min. The absorbance of the solution was measured at 517 nm spectrophotometrically. BHA was used as the standard chemical for a calibration curve construction. The percentage of radical inhibition was calculated using Equation 1. The results are expressed as effective concentration at 50% inhibition (IC50).

$$\text{Inhibition (\%)} = \frac{A_O - A_S}{A_O} \; x \; 100 \qquad (1)$$

Where A_o = absorbance of control and A_s = absorbance of sample.

2.6. —Cation radical scavenging activity

The cation radical inhibition of sample was determined using ABTS (2,2'azinobis(3-ethylbenzothiozoline-6-sulfonic acid) disodium salt) assay according to the method described by Biskup et al. (2013) with some modifications. The $ABTS^{\bullet+}$ solution was prepared by reacting ABTS (7 mM) with potassium persulfate (2.45 mM) at a ratio of 1:1, and incubated overnight in a dark place. The solution was then diluted with 50% methanol to have an absorbance of 1.00 at 734 nm. Samples were also dissolved in 50% methanol in the concentration of 0 to 1,000 mg/mL. Then, 2 mL of the diluted $ABTS^{\bullet+}$ was added with 100 µL sample solution, and incubated for 6 min under subdued light condition. The absorbance was measured at 734 nm using a UV-Vis spectrophotometer.

2.7. —Reducing power

The reducing power of samples was determined using ferric reducing antioxidant power (FRAP) assay which was carried out according to the procedures reported by Chua et al. (2013) with modification. FRAP reagent was freshly prepared by mixing 2.5 mL 2,4,6-tripydyl-s-triazine complex (10 mM, Fe^{3+}-TPTZ) in hydrochloric acid (40 mM), 2.5 mL iron (III) chloride (20 mM, $FeCl_3$) and 25 mL acetate buffer (0.3 M, pH 3.6). The reagent solution was kept in the dark at 37°C before use. Sample (0.2 mL) was mixed with 1.8 mL FRAP reagent, and incubated at room temperature under subdued light condition for 10 min. The absorbance was measured at 593 nm using a UV-Vis spectrophotometer. Ascorbic acid (10 mg/L) was used as standard chemical.

2.8. —Compound screening by LC-MS/MS

A Liquid chromatography (Ultimate 3000; Dionex Corporation; Sunnyvale, CA, USA) integrated with a diode array detector (Dionex Ultimate 3000) and a tandem mass spectrometer (QSTAR Elite; AB Sciex; Foster City, CA, USA) was used for compound screening. Compounds were separated by a C18 XSelect HSS T3 column (2.1 mm × 100 mm, 2.5 µm) at a flow rate of 150 µL/min. A binary solvent system consisted of solvent A (water

with 0.1% formic acid) and solvent B (acetonitrile) was used as the mobile phase at the following gradient: 0–10 min, 10% B; 10–20 min, 10–80% B; 20–25 min, 80% B; 25–25.1 min, 80–10% B; and 25.1–30 min, 10% B. The injection volume was 5 µL. Compounds were eluted from the column and detected at the wavelength of 254 nm. Subsequently, compounds were ionized by a turbo ion spray (-4,500 V) before mass detection at the negative ion mode. The mass range was set at the range of 100–1000 m/z. Nitrogen gas was used for curtain gas (25 psi) and nebulizing gas (40 psi). The declustering potential was 40 V, whereas the focusing potential was 200 V. Samples were filtered using a 0.2 µm nylon membrane filter prior to injection.

2.9. Leaf morphological recognition

An in-house leaf image recognition system which was developed using the Java programming language was used to process the leaf images of both herbal species, namely *C. asiatica* and *H. verticillata*. The leaf image of each plant species was uploaded into the system for image processing and feature extraction. The leaf images were pre-processed via segmentation, grayscale conversion and noise removal. The key features such as leaf edge, vein pattern and dimension were extracted from the processed images using a serial of algorithms. Prewitt and thinning algorithms were used for edge detection. The algorithms of CheckLines, CheckLineLength, paintLines and paintPoints were used to construct the vein pattern of leaves. An array of tokens was designed to identify the coordinates of lines using cosinus and sinus angles for the determination of diagonal dimension.

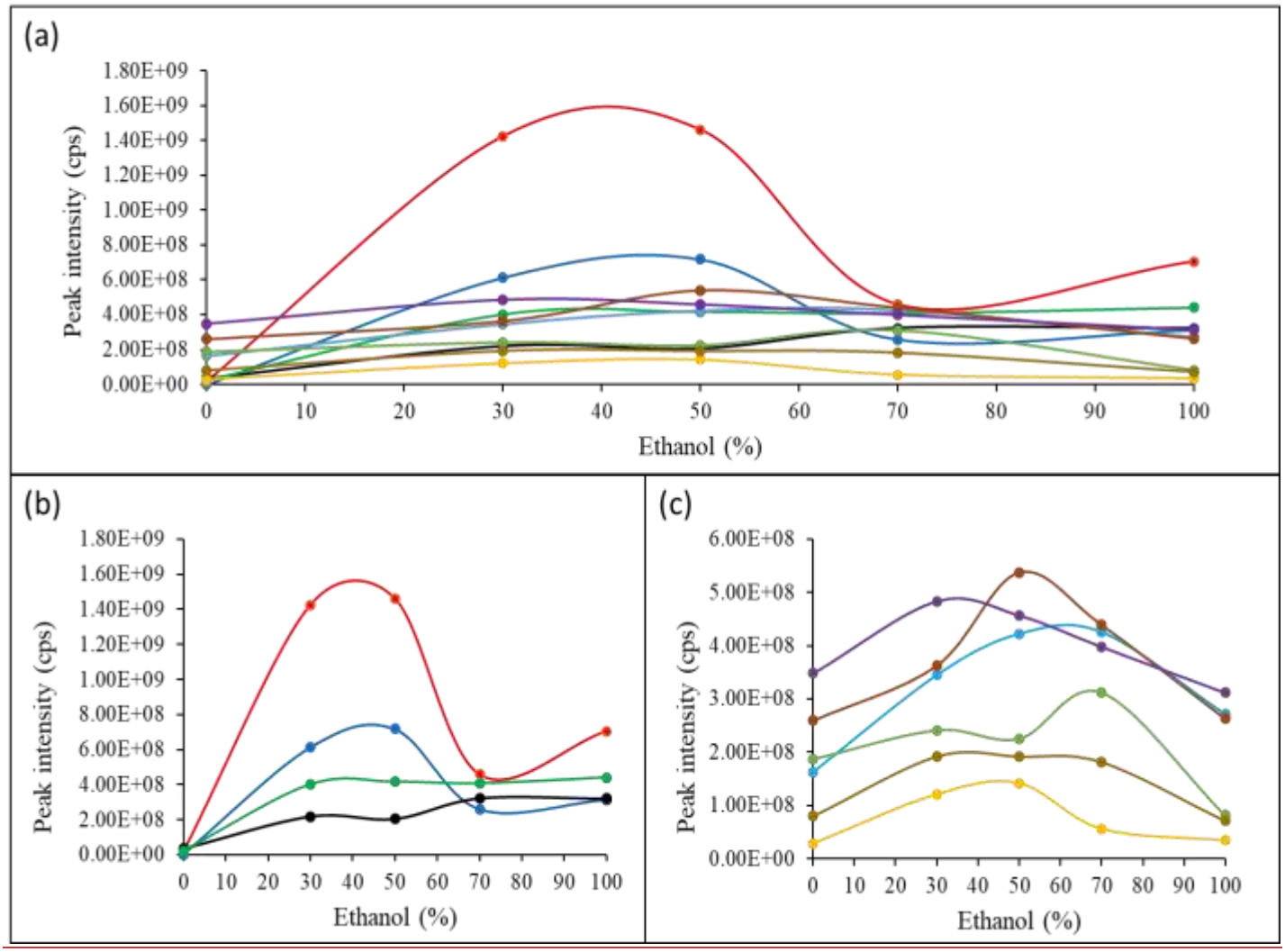
2.10. Multivariate data analysis

An unsupervised principal component analysis was carried out using a Pareto scaling in the data processing software (MarkerView 1.2.1, Applied Biosystems/MDSSciex, Foster City, CA, USA). The parameters for peak finding and alignment were set as minimum peak width, 0.05 Da; mass tolerance, 0.01 Da and retention time tolerance, 0.5 min.

3. Results and Discussion

3.1. High throughput mass screening

A high throughput mass screening was performed to detect phytochemicals in *C. asiatica* extracts which were prepared using different concentrations of ethanol ranged from 0-100%. The previously reported phytochemicals such as phenolic acids (caffeoylquinic acid, dicaffeoylquinic acid and dicaffeoyl methoxyoxaloylquinic acid), flavonoids (kaempferol, quercetin and glucuronyl quercetin) and triterpenoids (asiatic acid, madecassic acid, asiaticoside and madecasspside) were detected in this study. The intensities of the compound peaks are plotted in Figure 1. The figure shows that madecassic acid has the highest peak intensity, and followed by asiatic acid among the detected phytochemicals. The figure also shows that 50% ethanol is likely to be the most effective ethanol composition in the solvent system for the phytochemicals extraction.


(a)
Peak intensity (cps)
1.80E+09
1.60E+09
1.40E+09
1.20E+09
1.00E+09
8.00E+08
6.00E+08
4.00E+08
2.00E+08
0.00E+00
0 10 20 30 40 50 60 70 80 90 100
Ethanol (%)
(b)
Peak intensity (cps)
1.80E+09
1.60E+09
1.40E+09
1.20E+09
1.00E+09
8.00E+08
6.00E+08
4.00E+08
2.00E+08
0.00E+00
0 10 20 30 40 50 60 70 80 90 100
Ethanol (%)
(c)
Peak intensity (cps)
6.00E+08
5.00E+08
4.00E+08
3.00E+08
2.00E+08
1.00E+08
0.00E+00
0 10 20 30 40 50 60 70 80 90 100
Ethanol (%)

191

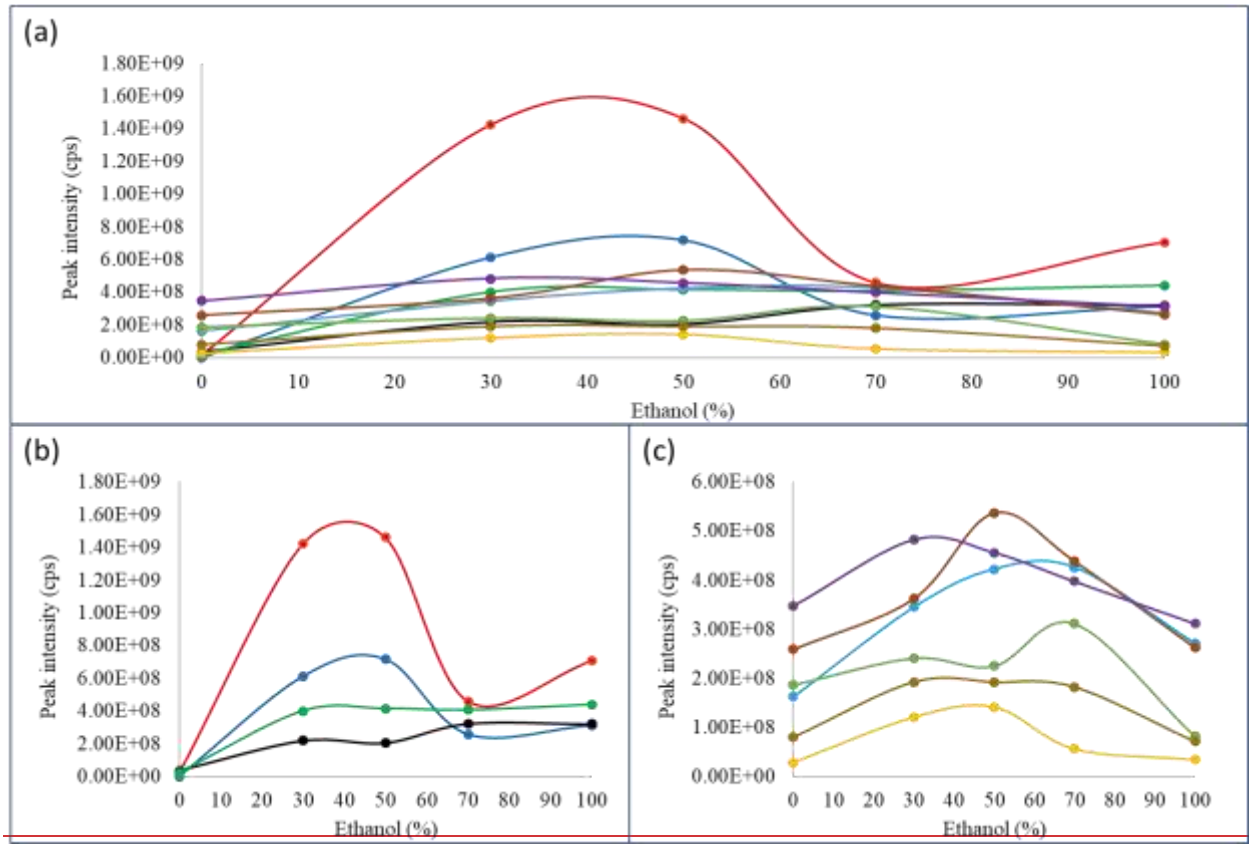


Figure 1. (a) Ten target phytochemicals consisted of (b) two triterpenoids and their trisaccharides and (c) three phenolic acids, two flavonoids and one glycosylated flavonoid in the extracts of *Centella asiatica* prepared using different ethanol concentrations, where asiatic acid, madecassic acid, asiaticoside, madecassoside, caffeoylquinic acid, dicaffeoylquinic acid, dicaffeoylmethoxyoxaloylquinic acid, quercetin, kaempferol and glucuronyl quercetin

In the subsequent analysis, 50% ethanolic extracts of the leaves and pericladial petioles of *C. asiatica* were examined for total phenolic, flavonoid and triterpenoid content spectrophotometrically (Figure 2). The results showed that leaf extract exhibited higher content of phytochemicals such as phenolics, flavonoids and triterpenoids than pericladial petiole extract. The proximate content of phytochemicals was also compared with its mimicking counterpart, *H. verticillata*. The comparison revealed that both herbal species had different compositions of phytochemicals, and phenolics was being the largest phytochemical group in the samples (Figure 2).

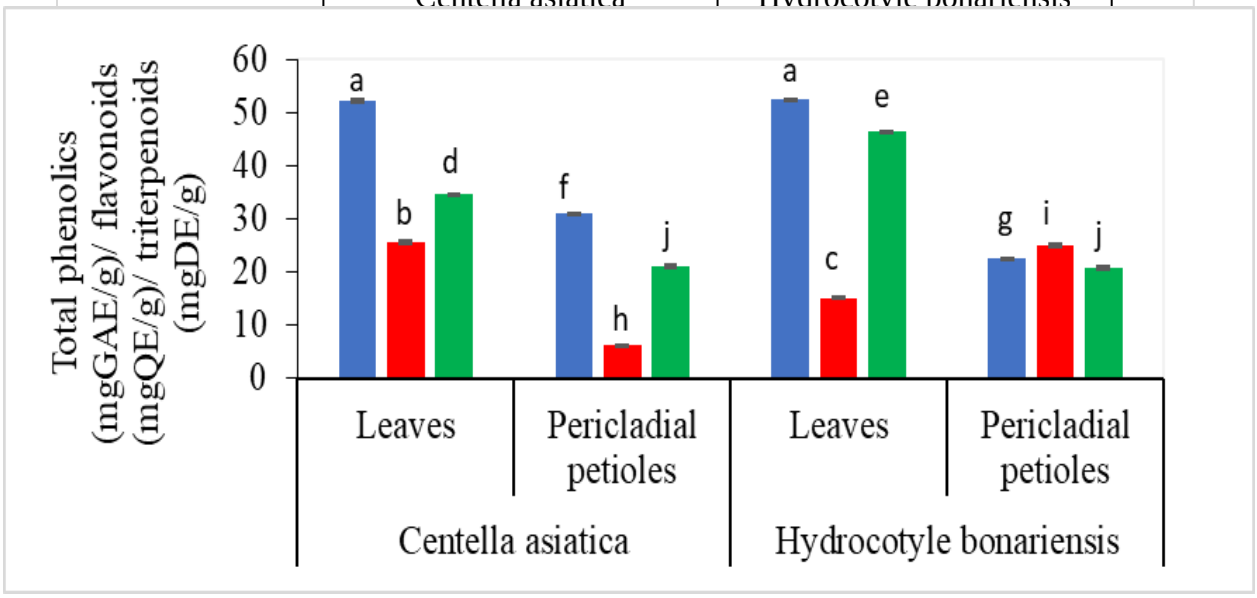


Figure 2. Total phenolics (blue bar), flavonoids (red bar) and triterpenoids (green bar) of the leaf and pericladial petiole extracts from *Centella asiatica* and *Hydrocotyle verticillata*. One-way analysis of variance (ANOVA) followed by T-test paired two samples for means were conducted to determine the significant difference of phytochemical content in the leaf samples of *C. asiatica* and *H. verticillate*, and in the pericladial petiole samples of *C. asiatica* and *H. verticillate*. Different small letters indicate the significant difference at $p < 0.05$.

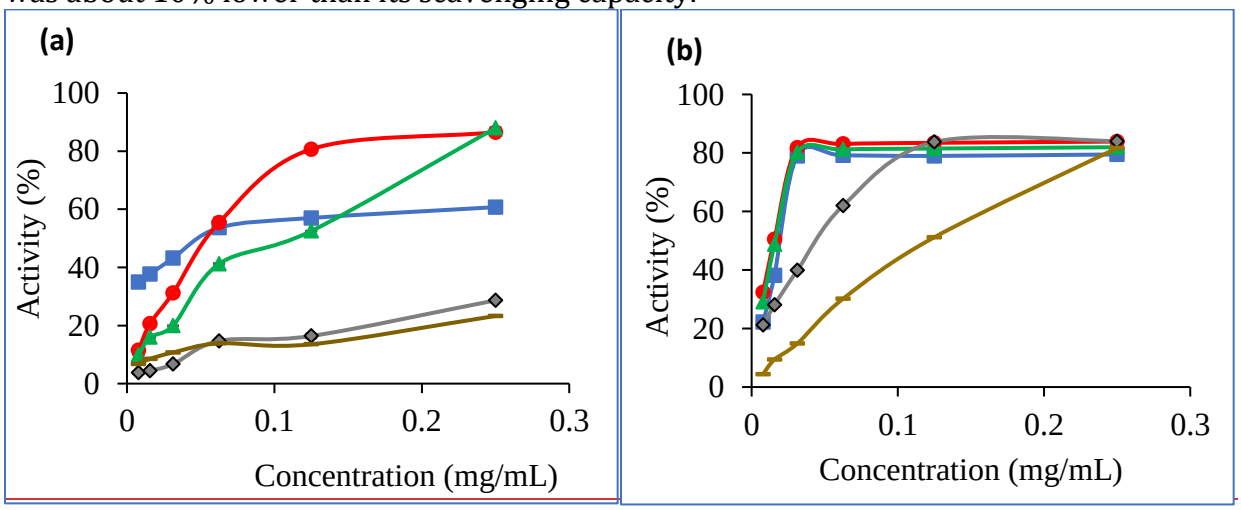
~~Analysis of variance was conducted to determine the significant difference of phytochemical content in the leaf (capital letter) and pericladial petiole (small letter) extracts of both herbs. Leaf extracts with different capital letter or pericladial petiole extracts with different small letter indicate the significant difference at $p < 0.05$.~~

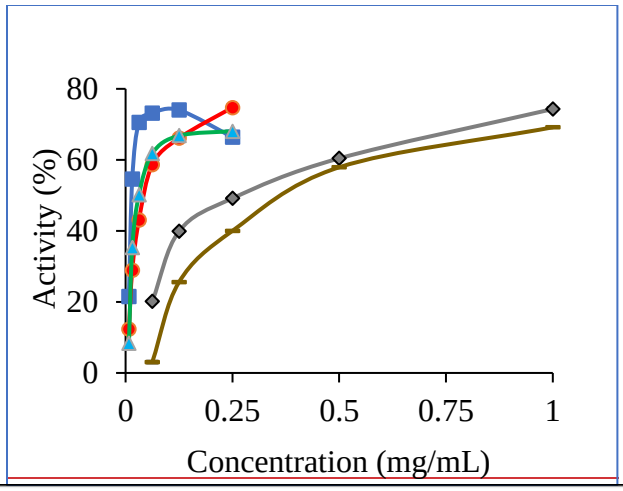
Total phenolic content was determined using the widely accepted Folin-Ciocalteu assay. This assay is a non-specific phenol oxidation in alkaline medium catalyzed by two strong inorganic oxidants, namely phosphotungstic and phosphomolibdic acids. The heteropoly

acid was reduced from the valence state of +6 to +5, and resulting the formation of blue molybdenum-tungsten complex for absorbance measurement. The other non-phenolic organic and inorganic compounds could possibly contribute to an elevated apparent phenolic content. Hence, the assay actually describes the total reducing capacity of a sample which is often correlated to its antioxidant activity.

In the present study, quercetin was used as a standard chemical to build the calibration curve of total flavonoid content. The absorbance was attributed to the formation of acid labile complexes after chelating flavonoids with aluminum ions. Possibly, the C-4 keto, C-3 or C-5 hydroxyl groups and ortho-dihydroxyl groups in the A or B rings of flavonoids may chelate with aluminum ions to produce colored complex for detection (Kasprzak, et al., 2015). The use of aluminum ions in the presence of acetate salt was more suitable for flavonols (Pekal & Pyrzynska, 2014).

The antioxidant capacity of the herbal extracts was also evaluated in terms of scavenging free and cation radicals, as well as reducing ferric ions as presented in Figure 3. In line with the proximate content of phytochemicals, the antioxidant capacity of leaf extract was higher than its pericladial petiole extract. This is because the antioxidant capacity of plant extract is mostly attributed to the presence of phytochemicals, particularly phenolic acids and flavonoids. The figure also clearly shows that the leaf extract of *C. asiatica* could exhibit the highest scavenging activities against free and cation radicals, and reducing power. The 50% ethanolic extract was also found to be an effective radical scavenger compared to its capacity as a reducing agent. This was because the concentration of extract which was required to inhibit 50% of radicals was lower than that value to reduce ferric ions. The scavenging activity could achieve more than 80%, whereas the reducing power was about 70% which was about 10% lower than its scavenging capacity.




Activity (%)
Concentration (mg/mL)

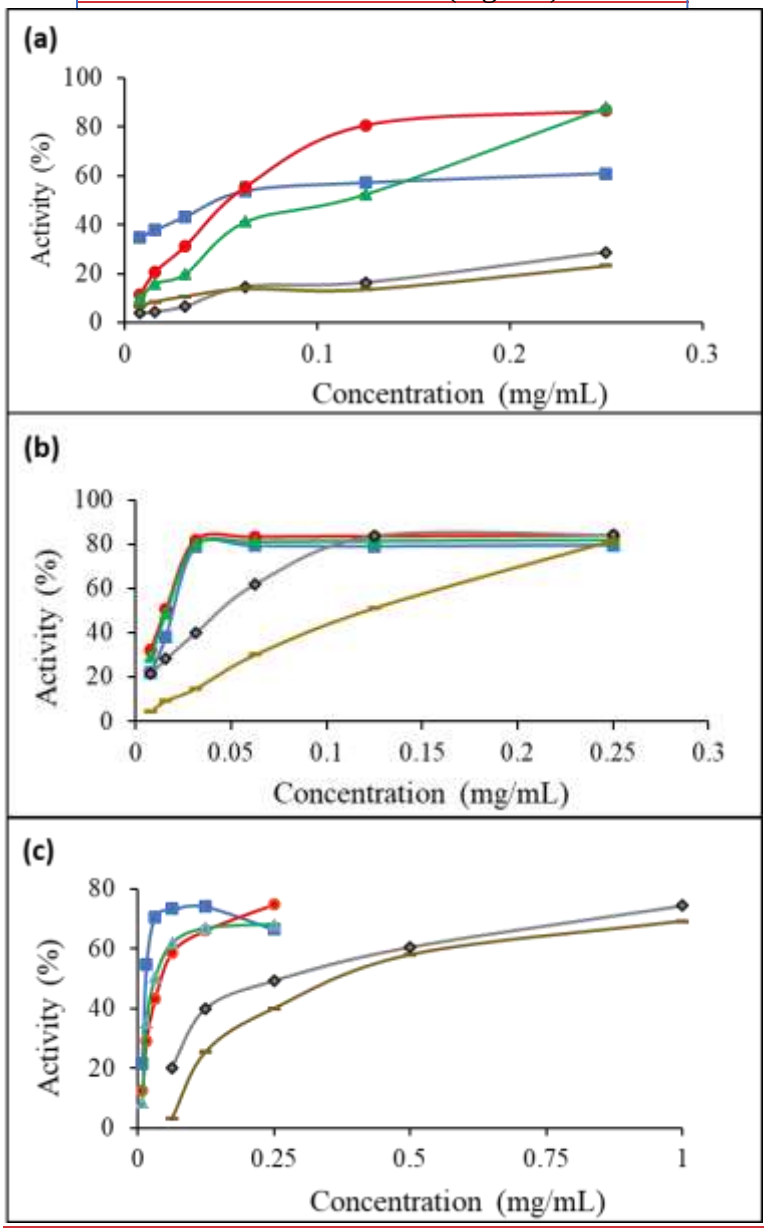

(a)
Activity (%)
Concentration (mg/mL)
(b)
Activity (%)
Concentration (mg/mL)
(c)
Activity (%)
Concentration (mg/mL)

Figure 3. Antioxidant capacity of extracts based on the scavenging activities of (a) free and (b) cation radicals, as well as (c) reducing power of ferric ions for ascorbic acid (■), the leaf extract of *Centella asiatica* (●), the leaf extract of *Hydrocotyle verticillate* (▲), the pericladial petiole of *C. asiatica* (♦) and the pericladial petiole of *H. verticillate* (−).

The antioxidant compounds primarily follow the electron transfer mechanism to inhibit the radicals. The compounds might also involve in hydrogen atom transfer at a slower rate (Gulcin, 2020). Therefore, compounds with bulky rings would have the difficulty to access radicals for electron transfer. On the other hand, compounds with conjugated double bonds and multiple hydroxyl groups would be the dominant chemical characteristics to inhibit radicals. DPPH assay is considered to be more selective because aromatic acid with a single hydroxyl group does not react with DPPH radicals (Cerretani & Bendini, 2010). This also indicates that the leaf extract of *C. asiatica* may have many polyol phenolics either from the group of phenolic acids or polyphenols.

Compounds react with ABTS radicals would also respond to the FRAP assay because of similarity in redox potentials (Gulcin, 2020). However, the results showed to have higher concentration of samples to inhibit 50% of ferric ions. The lower reducing power could only be contributed by water soluble antioxidative compounds (Apak, et al., 2007). The acidic medium of FRAP assay was used to promote ferric ion solubility which indirectly increased the redox potential. Pulido et al. (2000) reported that the absorbance of compounds such as caffeic acid, quercetin and tannic acid was not stabilized even after several hours of reaction time in FRAP assay. The observation was in good agreement with previous researchers that antioxidant activity measured in FRAP assay was lower than that in ABTS assay (Gulcin, 2020).

The variance of phytochemicals in both herbs could be clustered into 3 major principal components. The unsupervised multivariate analysis indicated that the phytochemicals in both herbs could achieve up to 78.4 % of the total variance for the first principal component (PC1). Figure 4 shows the phytochemicals in *C. asiatica* are prone to be located at the positive region, whereas the phytochemicals in *H. hydrocotyle* are mostly located at the negative region of PC1. The phytochemicals such as m/z 301 (quercetin), 353 (caffeoylquinic acid), 609 (glucosylrhamnosyl quercetin or rutin), 721 (tricaffeoyl-2,7-anhydro-2-octulopyranosonic acid) and 1101 (saponin) are likely to be the dominant compounds differentiating *H. hydrocotyle* from *C. asiatica* (Figure 4(c)). Although m/z 461 (unknown), 477 (glucuronyl quercetin), 515 (glycosyl caffeoylquinate) and 601 (dicaffeoyl methoxyoxaloylquinic acid) were found in both plant species, they were present in higher amount in *C. asiatica* (Figure 4(d)). The pentacyclic triterpenoids and their trisaccharides were located near the center of the axis as indicated in Figure 4.

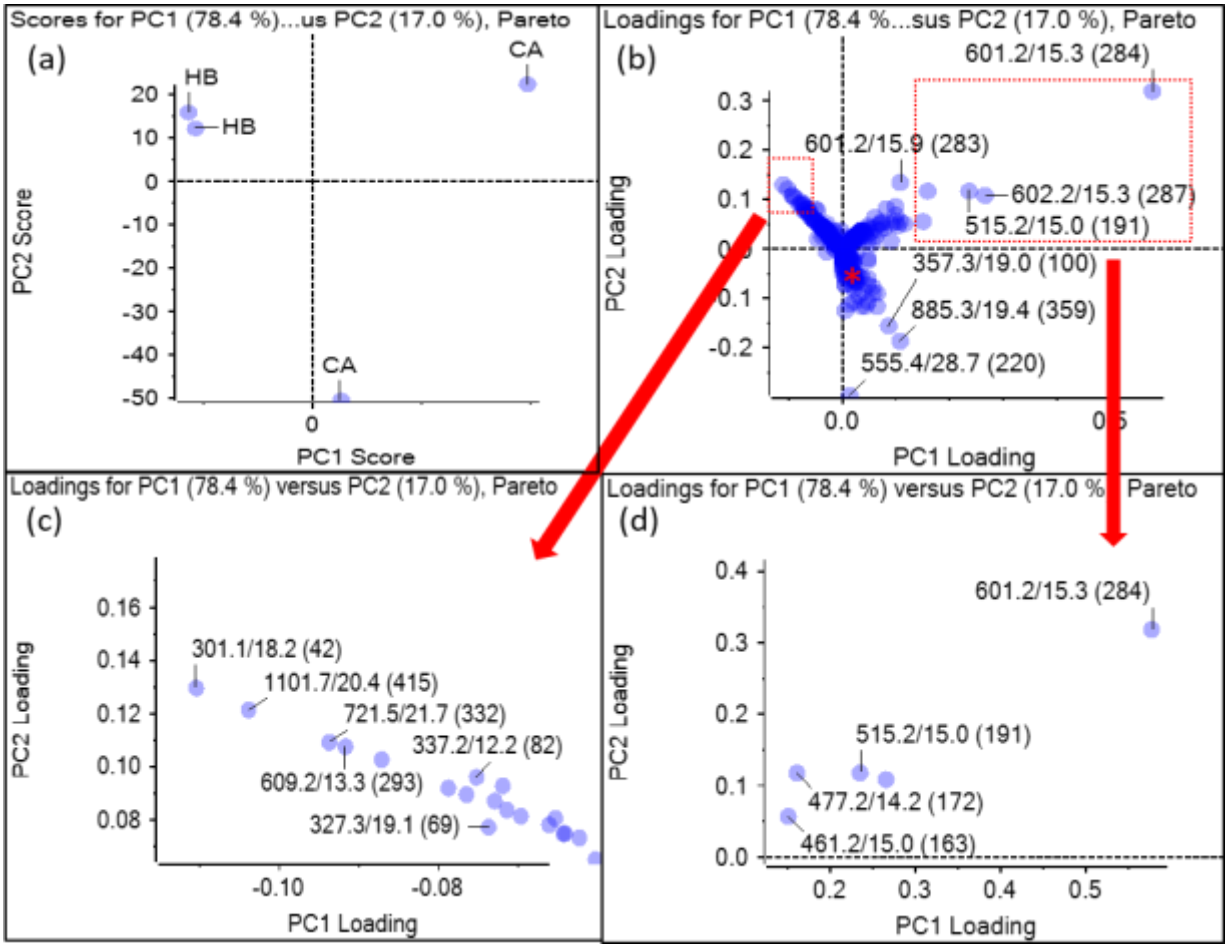


Figure 4. (a) Score and (b) loading plots of *Centella asiatica* (CA) and *Hydrocotyle verticillata* (HB) with the zoom-in area of masses, specifically for (c) HB in the negative region and (d) CA in the positive region of first principal component. * is the location of the pentacyclic triterpenoids and their trisaccharides in *C. asiatica*.

3.2. —Comparison of target phytochemicals

The presence of selected phytochemicals was then compared in both 50% ethanolic extracts of *C. asiatica* and *H. verticillata*. The comparison is made in term of its peak intensity as presented in Figure 5 (supplementary). The figure clearly illustrates that *C. asiatica* has higher content of the target phytochemicals, except for caffeoylquinic acid and quercetin. This could support the belief that *C. asiatica* is more active for ethnomedicine, especially for gastrointestinal disorders like dysentery, constipation, stomach problems, indigestion and loss of appetite, and for memory enhancement (Jahan, et al., 2012). Interestingly, there were a few of glycosylated polyphenols detected only in the extract of *H. verticillata* as listed in Table 1 (Supplementary). The quick mass screening results indicated that *C. asiatica* had higher triterpenoids and their glycosides, whereas *H. verticillata* contained more polyphenols and their glycosides. Previous researchers from Taiwan also reported the

detection of quercetin, isorhamnetin and rutin in Hydrocotyle species (Huang, et al., 2008; Yang, et al., 2008). The results revealed that both species are totally different in phytochemical profile, even they are locally called as Pegaga. The difference in phytochemical profile most possibly will contribute to pharmacological variance.

3.3. Differentiation of leaf morphology

The leaf images of both plant species were also processed using the established computing system for comparison. This is one of the non-destructive and rapid recognition techniques for plant recognition. The leaf edge including shape, vein pattern and dimension are selected as the dominant leaf features for the differentiation of plant species (Ehsani Rad, 2010; Lee & Hong, 2013). The leaf colour was not considered because this feature might be changed due to the seasonal and environmental factors.

The edge of plant leaves is the most obvious and easily recognised feature for identification. Prewitt algorithm was used to detect the edge of leaves in this study. This algorithm has been proven for its reliability for the leaf classification and plant disease detection in previous studies. (Navarajan, et al., 2015; Vilasini Ramamoorthy, 2020). The detected edge points produced pixels forming the leaf edge and vein as presented in Figure 6 (supplementary). From the pixels produced by Prewitt algorithm, it is clearly indicated that both species of plants have different shapes and vein patterns morphologically. The leaves of *C. asiatica* show to have kidney shape with second order veins branched off at the intervals of several first order veins, and reticulate meshes could also be observed between the third order veins and minor veins. On the other hand, the round shaped leaf of *H. verticillata* displays multiple first order veins.

Vein pattern could be the fingerprint of plants which is sometimes not easily observed without the assistance of pattern recognition tool (Scoffoni, et al., 2008). Therefore, the use of high performance computing system would be the method of choice. Besides phytochemical identification, leaf morphology including the vein pattern has been recognized as a reliable tool in identifying plant species. In the present study, both *C. asiatica* and *H. verticillata* belong to palmately veined species with multiple first order veins branching from the petiole (Sack, et al., 2008). The venation architecture is important to determine the sensitivity hydraulic conductance of leaves. A clear correlation has been established between the vein characteristics and properties of leaves, particularly on the aspects of leaf damage and drought tolerance (Scoffoni, et al., 2011; Sack, et al., 2008).

4. Conclusion

It is important to highlight that the difference of phytochemicals in *C. asiatica* and *H. verticillata*, even though both species are known as Pegaga in Malaysia. The findings of the study proved that *C. asiatica* contained pentacyclic triterpenoids (asiatic acid and madecassic acid) and their trisaccharides (asiaticoside and madecassocide), whereas *H. verticillata* contained high amount of quercetin and its glycosylated derivatives. The

different venation of the plant leaves has also explained the variance of phytochemical profiles which would contribute to different biological activities.

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Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

~~**Declaration of Competing Interest**~~

~~All authors listed have contributed sufficiently to the project to be included as authors. To the best of our knowledge, no conflict of interest, financial or other, exists.~~

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Supplementary

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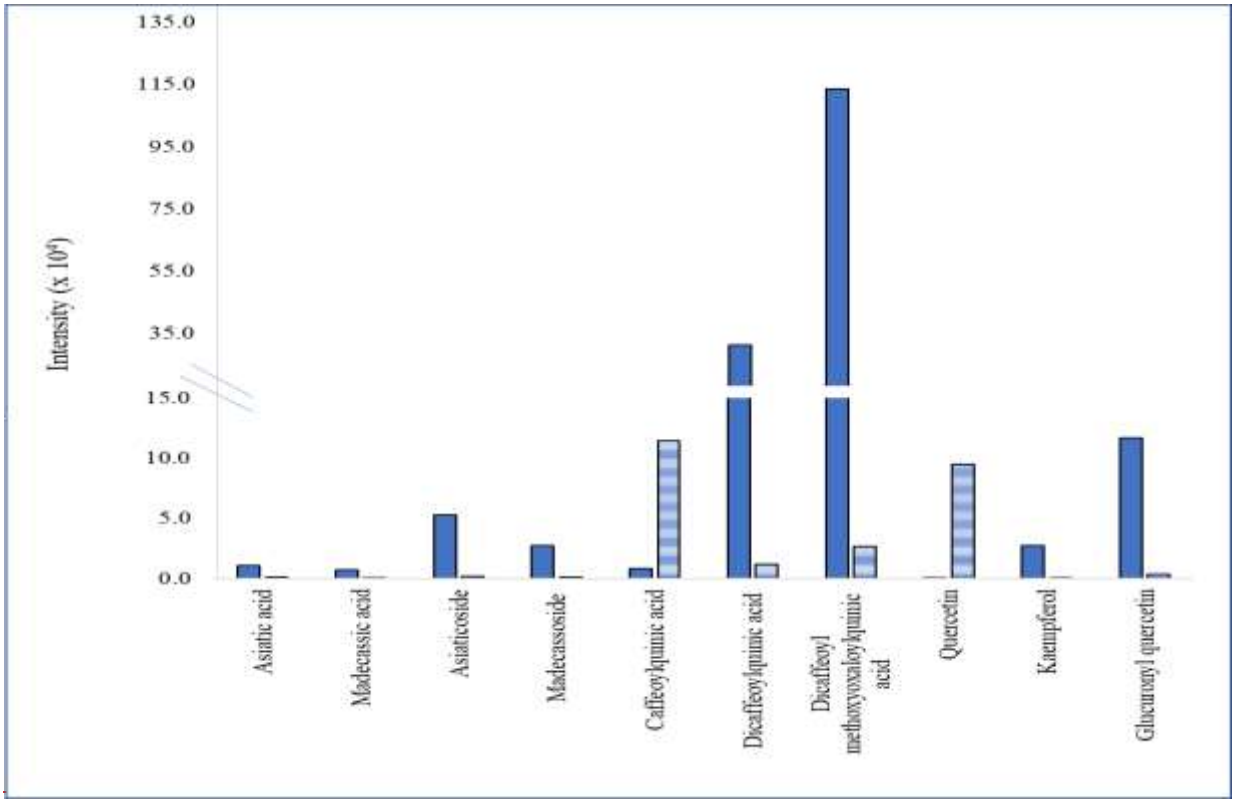
Intensity (x 10⁶)
135.0
115.0
95.0
75.0
55.0
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10.0
5.0
0.0
Asiatic acid
Madecassic acid
Asiaticoside
Madecassoside
Caffeoylquinic acid
Dicaffeoylquinic acid
Dicaffeoyl methoxyvanleoylquinic acid
Quercetin
Kaempferol
Glucuronyl quercetin

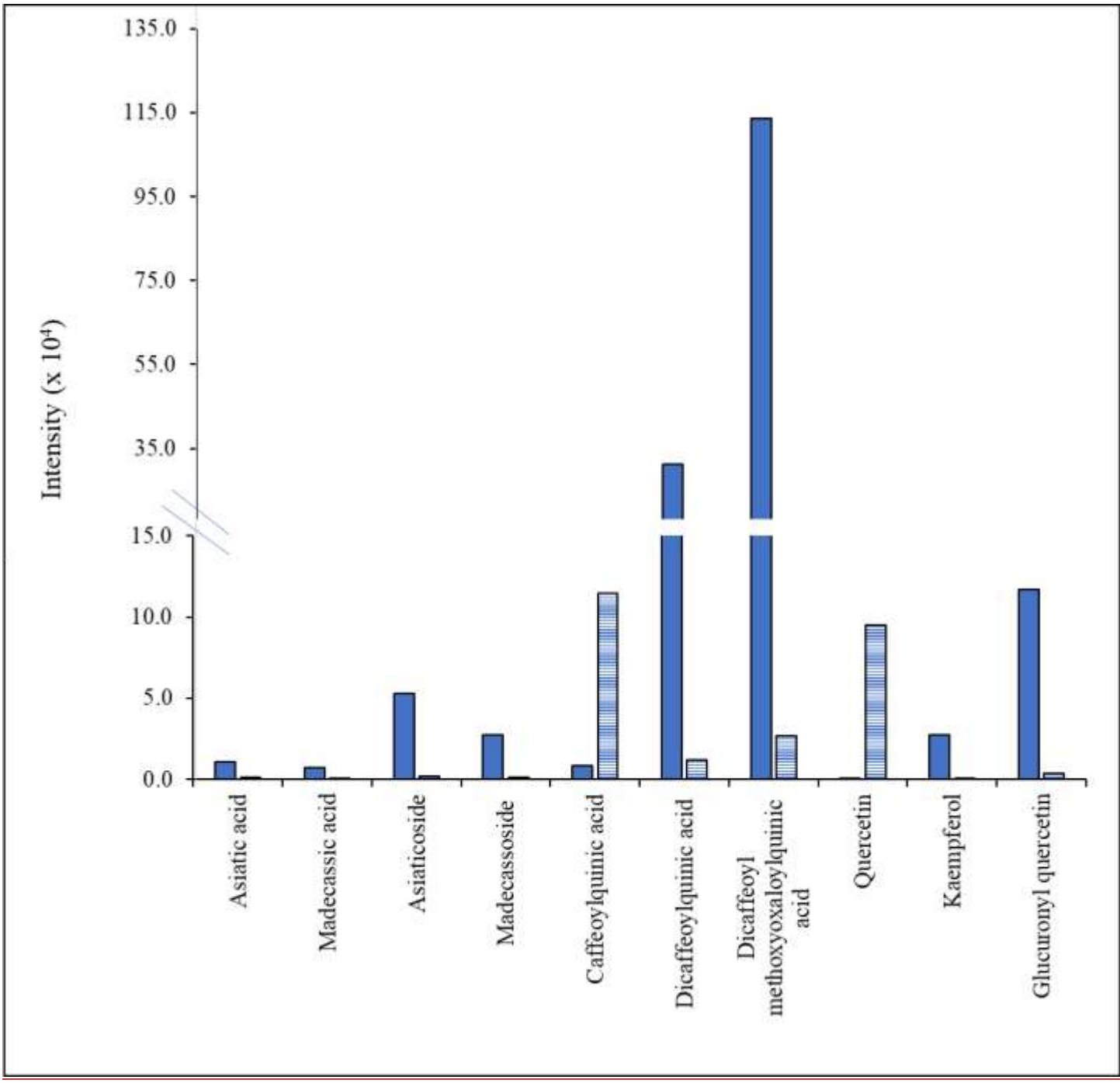

Figure 5 (supplementary). Target phytochemicals detected in *Centella asiatica* (solid blue bar) and *Hydrocotyle verticillata* (line blue bar) extracts prepared using 50% ethanol.

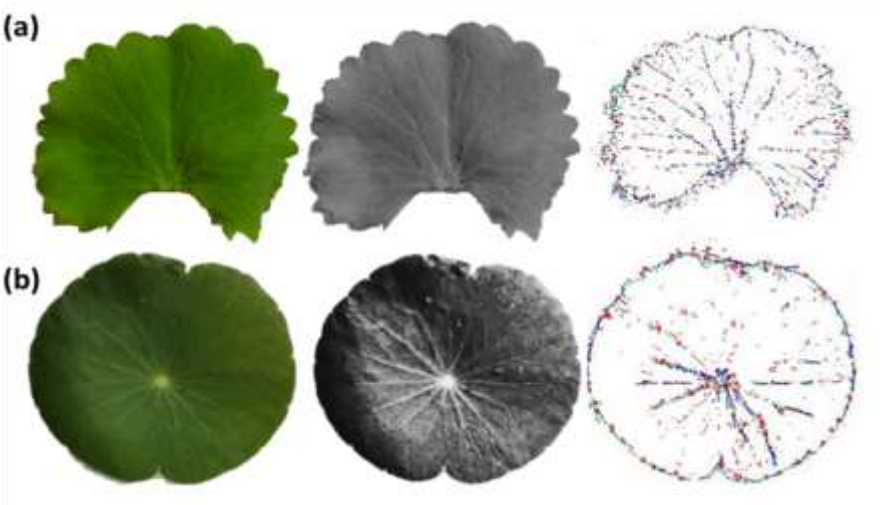

Figure 6 (supplementary). Original leaf morphological images of (a) *Centella asiatica* and (b) *Hydrocotyle verticillata* which are converted to gray scale and pixel images

Table 1 (supplementary). Phytochemicals detected in *Hydrocotyle verticillata* extract

Hydrocotyle verticillata	**Putative compounds**	**References**
301/273/179/151	quercetin	(Maulidiani, et al., 2014)
433/300(-133)/299/271	quercetin pentoside	(Maulidiani, et al., 2014)
447/300(-147)/283/271(-176)/255	isorhamnetin pentoside	(Li, et al., 2016)
463/300(-163)/271(-192)	quercertin glucoside	(Li, et al., 2016)
593/564/531(-62)/491/449/429/284/283(-310)/255/227	luteolin rutinoside	(Brito, et al., 2014)
609/507/361/300/271	rutin	(Maulidiani, et al., 2014)
639/463(-176)/300(-163)/269/255	caffeoyl rhamnocitrin glucuronide	(Chen, et al., 2016)
653/299(-354)/284	caffeoylquinoyl rhamnocitrin	(Chen, et al., 2016)
669/463(-206)/300(-369)/271(-398)/255	feruyl rhamnocitrin glucuronide	(Chen, et al., 2016)
695/300/299(-396)	rhamnocitrin tripentosides	(Chen, et al., 2016)
755/299bp(-456)/271(-484)	rhamnocitrin diglysoypentoside	(Chen, et al., 2016)
1187/581/285(-296)	$[2M-H]^-$, luteolin rutinoside	(Brito, et al., 2014)

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