



Telusuri dalam email



- 99+
- Tulis
- Mail
- Kotak Masuk 3.557
- Chat
- Berbintang
- Ditunda
- Spaces
- Penting
- Terkirim
- Meet
- Draf 20
- Kategori
- Selengkapnya

Label

Fwd: [FOFJ] 564 Paper Accepted with Major Revisions Eksternal



CHUA LEE SUAN FKK <chualeesuan@utm.my>
kepada saya

24 Mei 2023, 15.08 (13 jam y

----- Forwarded message -----

From: **CHUA LEE SUAN FKK** <chualeesuan@utm.my>
Date: Sun, May 14, 2023 at 10:33 PM
Subject: Fwd: [FOFJ] 564 Paper Accepted with Major Revisions
To: <ekasari_gt@yahoo.com>

----- Forwarded message -----

From: **CHUA LEE SUAN FKK** <chualeesuan@utm.my>
Date: Tue, May 24, 2022 at 8:22 PM
Subject: Re: [FOFJ] 564 Paper Accepted with Major Revisions
To: [FOFJ] Editorial Office <fofj_manage_editor@gmail.com>

Dear Professor,



Telusuri dalam email



99+

Tulis

Mail

Kotak Masuk 3.557

Chat

Berbintang

Ditunda

Spaces

Penting

Terkirim

Meet

Draf 20

Kategori

Selengkapnya

Label

----- Forwarded message -----

From: **CHUA LEE SUAN FKK** <chualeesuan@utm.my>

Date: Tue, May 24, 2022 at 8:22 PM

Subject: Re: [FOFJ] 564 Paper Accepted with Major Revisions

To: [FOFJ] Editorial Office <fofj_manage.editor@gmail.com>

Dear Professor,

Thank you for handling my manuscript and giving me the opportunity to publish my manuscript in the journal of Future of Foc Agriculture and Society.

We have revised the manuscript accordingly based on the comments given.

Herewith I attach the files below for your perusal.

- 1). Cover letter
- 2). Full manuscript with track change
- 3). Full manuscript (clean)
- 4). Zip file of all figures and table

Thank you very much.

Regards,
Chua LS



Telusuri dalam email



99+

Tulis

Mail

Kotak Masuk 3.557

Chat

Berbintang

Ditunda

Spaces

Penting

Terkirim

Meet

Draf 20

Kategori

Selengkapnya

Label

On Mon, May 23, 2022 at 5:28 PM [FOFJ] Editorial Office <fofj_manage_editor@gmail.com> wrote:

Dear author(s)

Your paper has been reviewed by two experts. Your manuscript was accepted with **major revisions**.

Please find attached the review reports, plagiarism test and the checklist. You need to read the checklist carefully to be aware of the steps to revise your manuscript. **Please read it slowly and carefully and do not miss or skip any required point.**

Please note that if the requirements in the checklist have NOT been fulfilled completely and accurately point by point, we will not be processing the paper even if it was accepted by reviewers.

Thank you for your cooperation and comprehension.

The deadline for re-submission is in 10 days.
Please do not hesitate to contact us if you have any questions or concerns.
Kind regards
Editorial office

--
Managing editor, Future of Food: Journal on Food, Agriculture and Society: <http://www.thefutureoffoodjournal.com/index.php/FOFJ>
University of Kassel
Nordbahnhofstr. 1a
37213 Witzenhausen
Germany



Telusuri dalam email



- 99+
- Tulis
- Mail
 - Kotak Masuk** 3.557
 - Berbintang
 - Ditunda
 - Penting
 - Terkirim
 - Draf** 20
 - Kategori**
 - Selengkapnya

Managing editor, Future of Food: Journal on Food, Agriculture and Society: <http://www.thefutureoffoodjournal.com/index.php/FOFJ>

University of Kassel
 Nordbahnhofstr. 1a
 37213 Witzenhausen
 Germany
 Universität Kassel
 Nordbahnhofstraße 1a
 37213 Witzenhausen
 Deutschland

Label

DISCLAIMER: The information in this e-mail and any attachment(s) transmitted with it ("Message") is intended only for the us and may contain confidential or privileged information. UTM are not responsible for any unauthorised changes made to the ir such changes. You are hereby notified that any action in reliance upon, or any review, retransmission, dissemination, distribu this Message or any part thereof by anyone other than the intended recipient(s) is strictly prohibited. Any opinions, conclusior this Message that do not relate to the official business of UTM shall be understood as neither given nor endorsed by UTM. U loss or damage caused by viruses transmitted by this Message.

2 Lampiran • Dipindai dengan Gmail





Telusuri dalam email



99+

Tulis

Mail

Kotak Masuk 3.557

Chat

Berbintang

Ditunda

Spaces

Penting

Terkirim

Meet

Draf 20

Kategori

Selengkapnya

Label

DISCLAIMER: The information in this e-mail and any attachment(s) transmitted with it ("Message") is intended only for the use and may contain confidential or privileged information. UTM are not responsible for any unauthorised changes made to the or such changes. You are hereby notified that any action in reliance upon, or any review, retransmission, dissemination, distribution this Message or any part thereof by anyone other than the intended recipient(s) is strictly prohibited. Any opinions, conclusions or this Message that do not relate to the official business of UTM shall be understood as neither given nor endorsed by UTM. U loss or damage caused by viruses transmitted by this Message.

2 Lampiran • Dipindai dengan Gmail



Balas

Teruskan

1 Article

2 Comparing herbal phytochemicals in different Pegaga: 3 *Centella asiatica* and *Hydrocotyle verticillata*

4 LEE SUAN CHUA^{1,2,*}, FARAH IZANA ABDULLAH² AND EKA SARI³

5 ¹Institute of Bioproduct Development, Universiti Teknologi Malaysia, 81310 UTM
6 Skudai, Johor Bahru, Johor, Malaysia.

7 ²Department of Bioprocess and Polymer Engineering, School of Chemical and Energy
8 Engineering, Faculty of Engineering, 81310 UTM Skudai, Johor Bahru, Johor, Malaysia.

9 ³International Institute of Aquaculture and Aquatic Sciences, Universiti Putra Malaysia,
10 71050 Sri Rusa, Port Dickson, Negeri Sembilan, Malaysia.

11 ⁴Bioengineering and Biomedical Engineering Laboratory, Research Centre of Sultan
12 Ageng Tirtayasa University, Serang, 42118 Banten, Indonesia.

13 *CORRESPONDING AUTHOR: chualesuan@utm.my; Tel.: +60-19-721-4378

14

15

16 Abstract:

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

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

32 1. Introduction

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

Future of Food Journal (FoFJ) Template

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

52 This herb has been widely used as folk remedies for thousands of years (Diwan, et al.,
53 1991). Recent publication also supports the beneficial use of the herb through scientific
54 studies. Scientists and researchers are getting interested to generate technical data in
55 line with the traditional remedies. The ever-increasing use of the herb has caused the
56 problem of adulteration purposely or unintentionally with cheaper material. The
57 common material that has been mistreated is *Hydrocotyle bonariensis* Comm. ex Lam,
58 which is usually called as largeleaf pennywort or coast pennywort from the plant family
59 Araliaceae (Plunkett, et al., 2004). This exotic aquatic macrophyte is also called as Ulam
60 Pegaga which means Pegaga salad in Malaysia. Similar phenomenon is happening in
61 Indonesia. The researchers reported that *C. asiatica* is potentially adulterated with
62 either *Hydrocotyle verticillata* or *Merremia emarginata* which have same local name as
63 Pegagan (Subositi, et al., 2016; Maruzy, et al., 2020). The misidentification has also been
64 happened in Philippines by local folks (Daminar & Bajo, 2013). *H. bonariensis* is
65 primarily planted in canals and water features for aesthetics and phytoremediation
66 (Strosnider, et al., 2011). The juice of the plant is traditionally prepared to treat fever,
67 colds, coughs, hepatitis, influenza, pruritus and sore throat, as well as headaches and
68 urinary problems (Sujanapal & Sankaran, 2016). In 2014, a group of researchers from
69 Singapore compared the vegetative differences of *C. asiatica* and *H. verticillata*. *H.*
70 *verticillate* which is also known as water pennywort or whorled marsh-pennywort, is
71 an exotic aquatic macrophyte that is commonly found in marshes. The difference
72 between both species, in term of phytochemicals is extremely limited in literature. The
73 difference of phytochemicals in both species is of great importance, especially for
74 herbal product formulation.

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

Future of Food Journal (FoFJ) Template

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

89 2. Materials and Methods

90 2.1. Phytochemical extraction

91 Phytochemical extraction was conducted using 1 g powdered leaves and stems in 100
92 mL solvent systems consisted of different concentrations of ethanol ranged from 0-100
93 %v/v. The mixture was refluxed at the boiling points of the solvent systems for 2 hours.
94 The supernatant was collected after centrifuged and filtered by Whatman cellulose
95 filter paper (Grade 1, 110 mm x 11 µm). The supernatant was then concentrated using
96 a rotary evaporator and dried in an oven at 50 °C until dryness. The weight of dried
97 crude extract was recorded. All experiments were carried out in triplicate, unless
98 otherwise stated.

99 2.2. Total Phenolic Content

100 The total phenolic content of samples was estimated using the colorimetric method
101 according to the procedures described by Siddiqui et al. (2017) with modification.
102 Different concentrations of samples were reconstituted in 50% methanol. About 1 mL
103 methanolic sample was mixed with 5 mL Folin–Ciocalteu reagent which were
104 previously diluted with deionized water. The mixture was left for 5 min at 25 °C and
105 then added with 5 mL sodium carbonate (7.5%). After incubation for 20 min, the
106 absorbance of the mixture was measured using a UV-Vis spectrophotometer (UV-1800,
107 Shimadzu, Japan) at 760 nm. A calibration curve of standard chemical, gallic acid (0 -
108 100 µg/mL) was constructed and the results are expressed as milligram gallic acid
109 equivalent per gram sample (mg GAE/g).

110 2.3. Total Flavonoid Content

111 The total flavonoid content of samples was also estimated using the colorimetric
112 method (Aryal, et al., 2019). An aliquot of 1 mL sample was mixed with 3 mL methanolic
113 AlCl₃ solution (10 %w/v), 0.2 mL potassium acetate (1 M) and 5.6 mL distilled water.



Future of Food Journal (FoFJ) Template

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

117 **2.4. Total triterpenoid content**

118 The total triterpenoid content was estimated spectrophotometrically using vanillin
119 assay (Chua, et al., 2019). The 1 mg/mL methanolic sample (250 µL) was added into a
120 test tube containing 8g/100 mL vanillin (250 µL) and topped up with 72 % sulfuric acid
121 (2.5 mL). The mixture of the solution was heated for 10 min at 60 °C, and subsequently
122 cooled in an ice-water bath for 5 min. The absorbance of the solution was recorded by
123 a UV-vis spectrophotometer at 544 nm. Diosgenin (5.7–71.4 mg/L) was used as the
124 standard chemical to build a calibration curve. The results are expressed as mg
125 diosgenin equivalent per g sample (mg DE/g).

126 **2.5. Free radical scavenging activity**

127 The antiradical capacity of samples was determined using DPPH (2,2-diphenyl-2-
128 picrylhydrazyl) assay as described by Chu et al. (2000). A 2 mL sample at different
129 concentrations ranged from 100-500 µg/mL was added into 2 mL methanolic DPPH
130 (0.1 mM) solution. The mixture was kept aside in a dark area for 30 min. The
131 absorbance of the solution was measured at 517 nm spectrophotometrically. BHA was
132 used as the standard chemical for a calibration curve construction. The percentage of
133 radical inhibition was calculated using Equation 1. The results are expressed as
134 effective concentration at 50% inhibition (IC50).

$$135 \text{ Inhibition (\%)} = \frac{A_o - A_s}{A_o} \times 100$$

136 (1)

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

138 **2.6. Cation radical scavenging activity**

139 The cation radical inhibition of sample was determined using ABTS (2,2'-azinobis(3-
140 ethylbenzothiozoline-6-sulfonic acid) disodium salt) assay according to the method
141 described by Biskup et al. (2013) with some modifications. The ABTS^{•+} solution was
142 prepared by reacting ABTS (7 mM) with potassium persulfate (2.45 mM) at a ratio of
143 1:1, and incubated overnight in a dark place. The solution was then diluted with 50%
144 methanol to have an absorbance of 1.00 at 734 nm. Samples were also dissolved in 50%
145 methanol in the concentration of 0 to 1,000 mg/mL. Then, 2 mL of the diluted ABTS^{•+}
146 was added with 100 µL sample solution, and incubated for 6 min under subdued light
147 condition. The absorbance was measured at 734 nm using a UV-Vis spectrophotometer.

148 **2.7. Reducing power**

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



Future of Food Journal (FoFJ) Template

152 mL 2,4,6-tripyridyl-s-triazine complex (10 mM, Fe³⁺-TPTZ) in hydrochloric acid (40 mM),
153 2.5 mL iron (III) chloride (20 mM, FeCl₃) and 25 mL acetate buffer (0.3 M, pH 3.6). The
154 reagent solution was kept in the dark at 37°C before use. Sample (0.2 mL) was mixed
155 with 1.8 mL FRAP reagent, and incubated at room temperature under subdued light
156 condition for 10 min. The absorbance was measured at 593 nm using a UV-Vis
157 spectrophotometer. Ascorbic acid (10 mg/L) was used as standard chemical.

158 **2.8. Compound screening by LC-MS/MS**

159 A Liquid chromatography (Ultimate 3000; Dionex Corporation; Sunnyvale, CA, USA)
160 integrated with a diode array detector (Dionex Ultimate 3000) and a tandem mass
161 spectrometer (QSTAR Elite; AB Sciex; Foster City, CA, USA) was used for compound
162 screening. Compounds were separated by a C18 XSelect HSS T3 column (2.1 mm × 100
163 mm, 2.5 μm) at a flow rate of 150 μL/min. A binary solvent system consisted of solvent
164 A (water with 0.1% formic acid) and solvent B (acetonitrile) was used as the mobile
165 phase at the following gradient: 0–10 min, 10% B; 10–20 min, 10–80% B; 20–25 min,
166 80% B; 25–25.1 min, 80–10% B; and 25.1–30 min, 10% B. The injection volume was 5
167 μL. Compounds were eluted from the column and detected at the wavelength of 254
168 nm. Subsequently, compounds were ionized by a turbo ion spray (-4,500 V) before mass
169 detection at the negative ion mode. The mass range was set at the range of 100–1000
170 m/z. Nitrogen gas was used for curtain gas (25 psi) and nebulizing gas (40 psi). The
171 declustering potential was 40 V, whereas the focusing potential was 200 V. Samples
172 were filtered using a 0.2 μm nylon membrane filter prior to injection.

173 **2.9. Leaf morphological recognition**

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

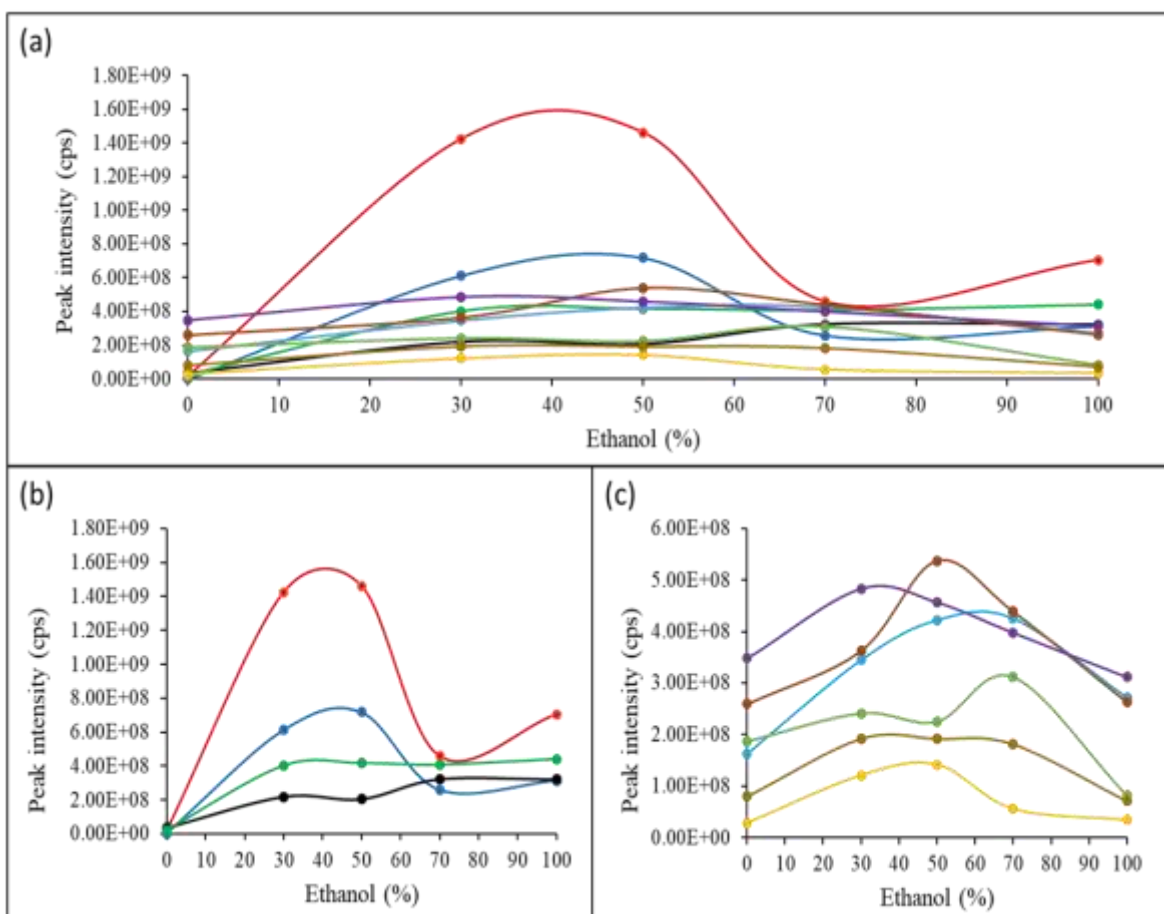
185 **2.10. Multivariate data analysis**

186 An unsupervised principal component analysis was carried out using a Pareto scaling
187 in the data processing software (MarkerView 1.2.1, Applied Biosystems/MDSSciex,
188 Foster City, CA, USA). The parameters for peak finding and alignment were set as
189 minimum peak width, 0.05 Da; mass tolerance, 0.01 Da and retention time tolerance,
190 0.5 min.

191 3. Results and discussion

192 3.1. High throughput mass screening

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



204

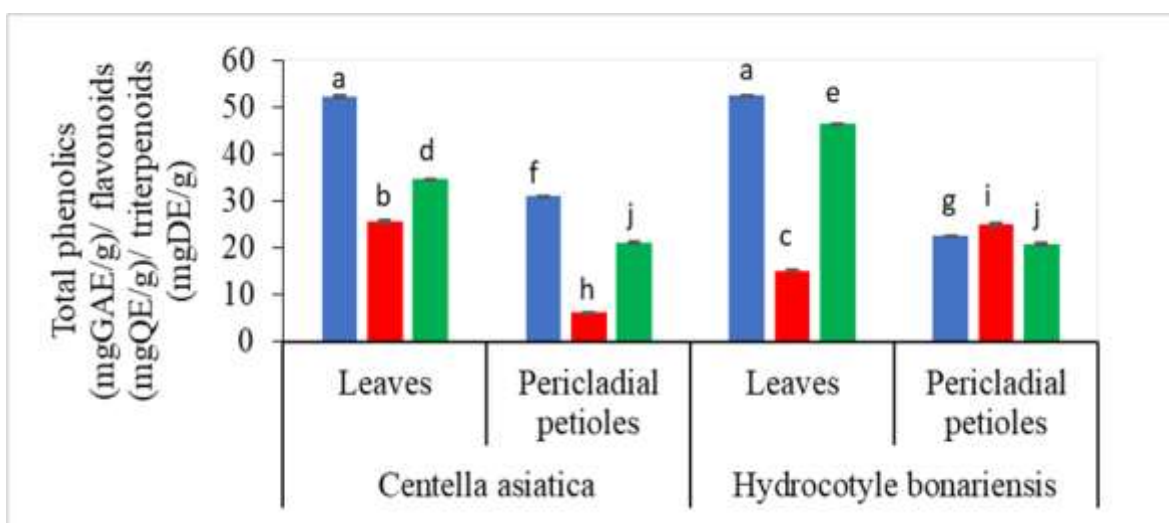
205 Figure 1. (a) Ten target phytochemicals consisted of (b) two triterpenoids and their
 206 trisaccharides and (c) three phenolic acids, two flavonoids and one glycosylated flavonoid in
 207 the extracts of *Centella asiatica* prepared using different ethanol concentrations, where ●
 208 asiatic acid,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

Future of Food Journal (FoFJ) Template

209 ● dicaffeoylquinic acid, ● dicaffeoylmethoxyoxaloylquinic acid, ● quercetin, ●
210 kaempferol and ● glucuronyl quercetin

211

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



220

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

227

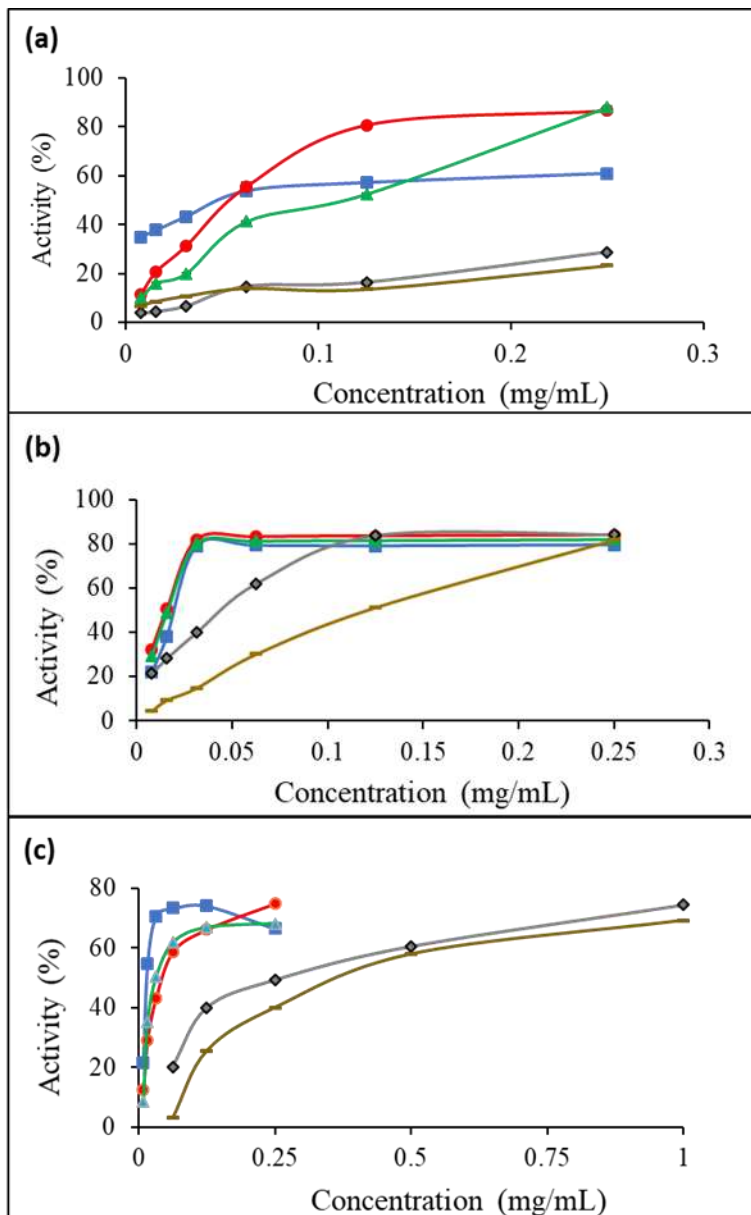
228 Total phenolic content was determined using the widely accepted Folin-Ciocalteu
229 assay. This assay is a non-specific phenol oxidation in alkaline medium catalyzed by two
230 strong inorganic oxidants, namely phosphotungstic and phosphomolibdic acids. The
231 heteropoly acid was reduced from the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



Future of Food Journal (FoFJ) Template

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

243 The antioxidant capacity of the herbal extracts was also evaluated in terms of
244 scavenging free and cation radicals, as well as reducing ferric ions as presented in
245 [Figure 3](#). In line with the proximate content of phytochemicals, the antioxidant capacity
246 of leaf extract was higher than its pericladial petiole extract. This is because the
247 antioxidant capacity of plant extract is mostly attributed to the presence of
248 phytochemicals, particularly phenolic acids and flavonoids. The figure also clearly
249 shows that the leaf extract of *C. asiatica* could exhibit the highest scavenging activities
250 against free and cation radicals, and reducing power. The 50% ethanolic extract was
251 also found to be an effective radical scavenger compared to its capacity as a reducing
252 agent. This was because the concentration of extract which was required to inhibit 50%
253 of radicals was lower than that value to reduce ferric ions. The scavenging activity could
254 achieve more than 80%, whereas the reducing power was about 70% which was about
255 10% lower than its scavenging capacity.



256

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

262

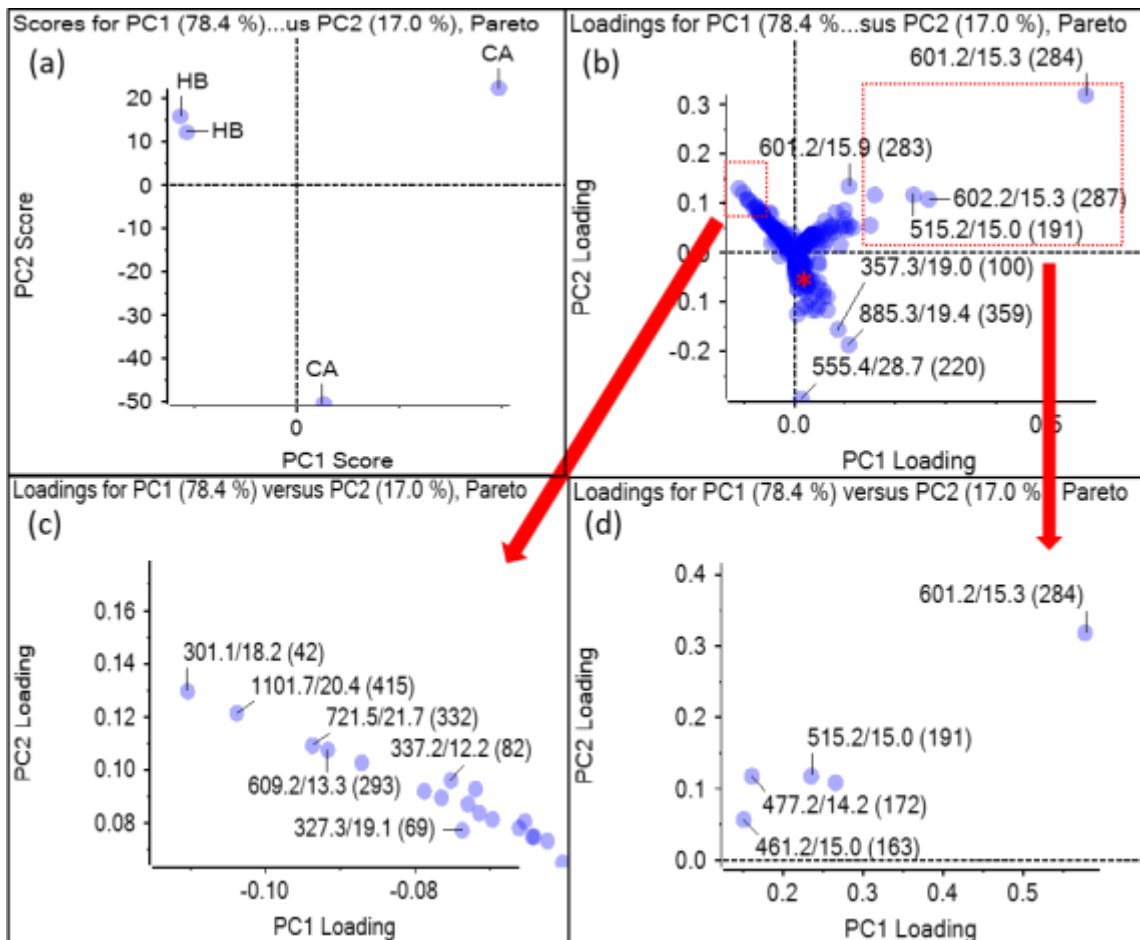
263 The antioxidant compounds primarily follow the electron transfer mechanism to inhibit
 264 the radicals. The compounds might also involve in hydrogen atom transfer at a slower
 265 rate (Gulcin, 2020). Therefore, compounds with bulky rings would have the difficulty
 266 to access radicals for electron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

Future of Food Journal (FoFJ) Template

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

272 Compounds react with ABTS radicals would also respond to the FRAP assay because of
273 similarity in redox potentials (Gulcin, 2020). However, the results showed to have
274 higher concentration of samples to inhibit 50% of ferric ions. The lower reducing power
275 could only be contributed by water soluble antioxidative compounds (Apak, et al.,
276 2007). The acidic medium of FRAP assay was used to promote ferric ion solubility
277 which indirectly increased the redox potential. Pulido et al. (2000) reported that the
278 absorbance of compounds such as caffeic acid, quercetin and tannic acid was not
279 stabilized even after several hours of reaction time in FRAP assay. The observation was
280 in good agreement with previous researchers that antioxidant activity measured in
281 FRAP assay was lower than that in ABTS assay (Gulcin, 2020).

282 The variance of phytochemicals in both herbs could be clustered into 3 major principal
283 components. The unsupervised multivariate analysis indicated that the phytochemicals
284 in both herbs could achieve up to 78.4 % of the total variance for the first principal
285 component (PC1). Figure 4 shows the phytochemicals in *C. asiatica* are prone to be
286 located at the positive region, whereas the phytochemicals in *H. hydrocotyle* are mostly
287 located at the negative region of PC1. The phytochemicals such as m/z 301 (quercetin),
288 353 (caffeoylquinic acid), 609 (glucosylrhamnosyl quercetin or rutin), 721 (tricafeoyl-
289 2,7-anhydro-2-octulopyranosonic acid) and 1101 (saponin) are likely to be the
290 dominant compounds differentiating *H. hydrocotyle* from *C. asiatica* (Figure 4(c)).
291 Although m/z 461 (unknown), 477 (glucuronyl quercetin), 515 (glycosyl
292 caffeoylquinic acid) and 601 (dicafeoyl methoxyoxaloylquinic acid) were found in both
293 plant species, they were present in higher amount in *C. asiatica* (Figure 4(d)). The
294 pentacyclic triterpenoids and their trisaccharides were located near the center of the
295 axis as indicated in Figure 4.



296

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

301

302 3.2. Comparison of target phytochemicals

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



Future of Food Journal (FoFJ) Template

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

319

320 3.3. Differentiation of leaf morphology

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

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

339 Vein pattern could be the fingerprint of plants which is sometimes not easily observed
340 without the assistance of pattern recognition tool (Scoffoni, et al., 2008). Therefore, the
341 use of high performance computing system would be the method of choice. Besides
342 phytochemical identification, leaf morphology including the vein pattern has been
343 recognized as a reliable tool in identifying plant species. In the present study, both *C.*
344 *asiatica* and *H. verticillata* belong to palmately veined species with multiple first order
345 veins branching from the petiole (Sack, et al., 2008). The venation architecture is
346 important to determine the sensitivity hydraulic conductance of leaves. A clear
347 correlation has been established between the vein characteristics and properties of
348 leaves, particularly on the aspects of leaf damage and drought tolerance (Scoffoni, et al.,
349 2011; Sack, et al., 2008).

350 5. Conclusions

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

358 **Acknowledgements**

359 This study was funded by Universiti Teknologi Malaysia (TDR-07G21-06G75 and HR-
360 08G84).

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.

361 **References**

362 Apak, R., Güçlü, K., Demirata, B., Ozyürek, M., Celik, S.E., Bektaşoğlu, B., Berker, K.I., &
363 Ozyurt, D. (2007). Comparative evaluation of various total antioxidant capacity assays
364 applied to phenolic compounds with the CUPRAC assay. *Molecules*, 12(7), 1496-1547.
365 [doi:10.3390/12071496](https://doi.org/10.3390/12071496)

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

369 Azlah, M.A.F., Chua, L.S., Rahmad, F.R., Abdullah, F.I., & Wan Alwi, S.R. (2019). Review
370 on Techniques for Plant Leaf Classification and Recognition. *Computers*, 8, 77.
371 [doi:10.3390/computers8040077](https://doi.org/10.3390/computers8040077)

372 Biskup, I., Golonka, I., Gamian, A., & Sroka, Z. (2013). Antioxidant activity of selected
373 phenols estimated by ABTS and FRAP methods. *Postępy Higieny i Medycyny*
374 *Doświadczalnej*, 67, 958-963. <http://www.phmd.pl/fulltxt.php?ICID=1066062>

375 Brinkhaus, B., Lindner, M., Schuppan, D., & Hahn, E.G. (2000). Chemical,
376 pharmacological and clinical profile of the East Asian medical plant *Centella asiatica*.
377 *Phytomedicine*, 7, 427-448. [doi:10.1016/S0944-7113\(00\)80065-3](https://doi.org/10.1016/S0944-7113(00)80065-3)

378 Brito, A., Ramirez, J.E., Areche, C., Sepúlveda, B., & Simirgiotis, M.J. (2014). HPLC-UV-MS
379 profiles of phenolic compounds and antioxidant activity of fruits from three citrus



Future of Food Journal (FoFJ) Template

- 380 species consumed in Northern Chile. *Molecules*, 19, 17400-17421.
381 [doi:10.3390/molecules191117400](https://doi.org/10.3390/molecules191117400)
- 382 Cerretani, L., & Bendini, A. (2010). Rapid assays to evaluate the antioxidant capacity of
383 phenols in virgin olive oil. *Olives and Olive Oil in Health and Disease Prevention*, 625-635.
384 [doi:10.1016/B978-0-12-374420-3.00067-X](https://doi.org/10.1016/B978-0-12-374420-3.00067-X)
- 385 Chaisawang, P., Sirichoat, A., Chaijaroonkhanarak, W., Pannangrong, W.,
386 Sripanidkulchai, B., Wigmore, P., & Welbat, J.U. (2017). Asiatic acid protects against
387 cognitive deficits and reductions in cell proliferation and survival in the rat
388 hippocampus caused by 5-fluorouracil chemotherapy. *PLoS ONE*, 12(7), e0180650.
389 [doi:10.1371/journal.pone.0180650](https://doi.org/10.1371/journal.pone.0180650)
- 390 Chen, G., Li, X., Saleri, F., & Guo, M. (2016). Analysis of flavonoids in *Rhamnus davurica*
391 and its antiproliferative activities. *Molecules*, 21, 1275.
392 [doi:10.3390/molecules21101275](https://doi.org/10.3390/molecules21101275)
- 393 Chu, Y., Chang, C., & Hsu, H. (2000). Flavonoid content of several vegetables and their
394 antioxidant activity. *Journal of the Science of Food and Agriculture*, 80, 561-566.
395 [doi:10.1002/\(SICI\)1097-0010\(200004\)80:5<561::AID-JSFA574>3.0.CO;2-%23](https://doi.org/10.1002/(SICI)1097-0010(200004)80:5<561::AID-JSFA574>3.0.CO;2-%23)
- 396 Chua, L.S., Lau, C.H., Chew, C.Y., & Dawood, D.A.S. (2019). Solvent fractionation and
397 acetone precipitation for crude saponins from *Eurycoma longifolia* extract. *Molecules*,
398 24, 1416. [doi:10.3390/molecules24071416](https://doi.org/10.3390/molecules24071416)
- 399 Chua, L.S., Rahaman, N.L.A., Adnan, N.A., & Tan, T.T.E. (2013). Antioxidant activity of
400 three honey samples in relation with their biochemical components. *Journal of*
401 *Analytical Methods in Chemistry*, 2013, 1-8. [doi:10.1155/2013/313798](https://doi.org/10.1155/2013/313798)
- 402 Daminar, N.L., & Bajo, L.M. (2013). Isolation and partial characterization of the most
403 bioactive metabolite from the hexane extract of the aerial part of *Hydrocotyle*
404 *verticillata* (whorled marshpennywort). *Global Journal of Science Frontier Research*,
405 13(2), 1-8.
- 406 Diwan, P.C., Karwande, I., & Singh, A.K. (1991). Anti-anxiety profile of mandukparni
407 (*Centella asiatica*) Linn in animals. *Fitoterapia*, 62, 255-257.
- 408 Ecology & Evolutionary Biology, Plant Biodiversity Conservatory and Research Core,
409 USA, Retrieved from <http://florawww.eeb.uconn.edu/ipm.html> (Accessed 26 June
410 2020).
- 411 Ehsani Rad, A. (2010). Plant Classification Based on Leaf Recognition. *International*
412 *Journal of Information Security*, 8, 77-81.
- 413 Gohil, K.J., Patel, J.A., & Gajjar, A.K. (2010). Pharmacological review on *Centella asiatica*:
414 a potential herbal cure-all. *Indian Journal of Pharmaceutical Sciences*, 72, 546-556.



Future of Food Journal (FoFJ) Template

- 415 [doi:10.4103/0250-474X.78519](https://doi.org/10.4103/0250-474X.78519)
- 416 Gulcin, İ. (2020). Antioxidants and antioxidant methods: an updated overview. *Archives*
417 *of Toxicology*, 94, 651–715. [doi:10.1007/s00204-020-02689-3](https://doi.org/10.1007/s00204-020-02689-3)
- 418 Huang, S.S., Huang, G.J., Ho, Y.L., Lin, Y.H., Hung, H.J./, Chang, T.N., Chang, M.J., Chen, J.J.,
419 & Chang, Y.S. (2008). Antioxidant and antiproliferative activities of the four *Hydrocotyle*
420 species from Taiwan. *Botanical Studies*, 49(4), 311-322.
- 421 Jahan, R., Hossain, S., Seraj, S., Nasrin, D., Khatun, Z., Das, P.R., Islam, M.T., Ahmed, I., &
422 Rahmatullah, M. (2012). *Centella asiatica* (L.) Urb.: Ethnomedicinal uses and their
423 scientific validations. *American-Eurasian Journal of Sustainable Agriculture*, 6(4), 261-
424 270.
- 425 Jamil, S.S., Nizami, Q., & Salam, M. (2007). *Centella asiatica* (Linn.) Urban: a review.
426 *Natural Product Radianc*, 6(2), 158–170.
427 <http://nopr.niscair.res.in/handle/123456789/7855>
- 428 Kasprzak, M.M., Erxleben, A., & Ochocki, J. (2015). Properties and applications of
429 flavonoid metal complexes. *Royal Society of Chemistry Advances*, 5, 45853-45877.
430 [doi:10.1039/C5RA05069C](https://doi.org/10.1039/C5RA05069C)
- 431 Kavitha, C.V., Agarwal, C., Agarwal, R., & Deep G. (2011). Asiatic acid inhibits pro-
432 angiogenic effects of VEGF and human gliomas in endothelial cell culture models. *PLoS*
433 *ONE*, 6(8), e22745. [doi:10.1371/journal.pone.0022745](https://doi.org/10.1371/journal.pone.0022745)
- 434 Lee, K.B., & Hong, K.S. (2013). An implementation of leaf recognition system using leaf
435 vein and shape. *International Journal of Bio-Science and Bio-Technology*, 5, 57–65.
- 436 Li, Z.H., Guo, H., Xu, W.B., Ge, J., Li, X., Alimu, M., & He, D.J. (2016). Rapid identification of
437 flavonoid constituents directly from PTP1B inhibitive extract of Raspberry (*Rubus*
438 *idaeus* L.) leaves by HPLC-ESI-QTOF-MS-MS. *Journal of Chromatographic Science*, 54(5),
439 05-810. [doi:10.1093/chromsci/bmw016](https://doi.org/10.1093/chromsci/bmw016)
- 440 Mangas, S., Moyano, E., Osuna, L., Cusido, R.M., Bonfill, M., & Palazo, J. (2008).
441 Triterpenoid saponin content and the expression level of some related genes in calli of
442 *Centella asiatica*. *Biotechnology Letters*, 30, 1853-1859. [doi:10.1007/s10529-008-9766-6](https://doi.org/10.1007/s10529-008-9766-6)
- 444 Maruzy, A., Budiarti, M., & Subositi, D. (2020). Authentication of *Centella asiatica* (L.)
445 Urb. (Pegagan) and its adulterant based on macroscopic, microscopic, and chemical
446 profiling. *Jurnal Kefarmasian Indonesia*, 10(1), 19-30. [doi:10.22435/jki.v10i1.1830](https://doi.org/10.22435/jki.v10i1.1830)
- 447 Maulidiani, Abas, F., Khatib, A., Shaari, K., & Lajis, N.H. (2014). Chemical characterization
448 and antioxidant activity of three medicinal Apiaceae species. *Industrial Crops and*
449 *Products*, 55, 238–247. [doi:10.1016/j.indcrop.2014.02.013](https://doi.org/10.1016/j.indcrop.2014.02.013)

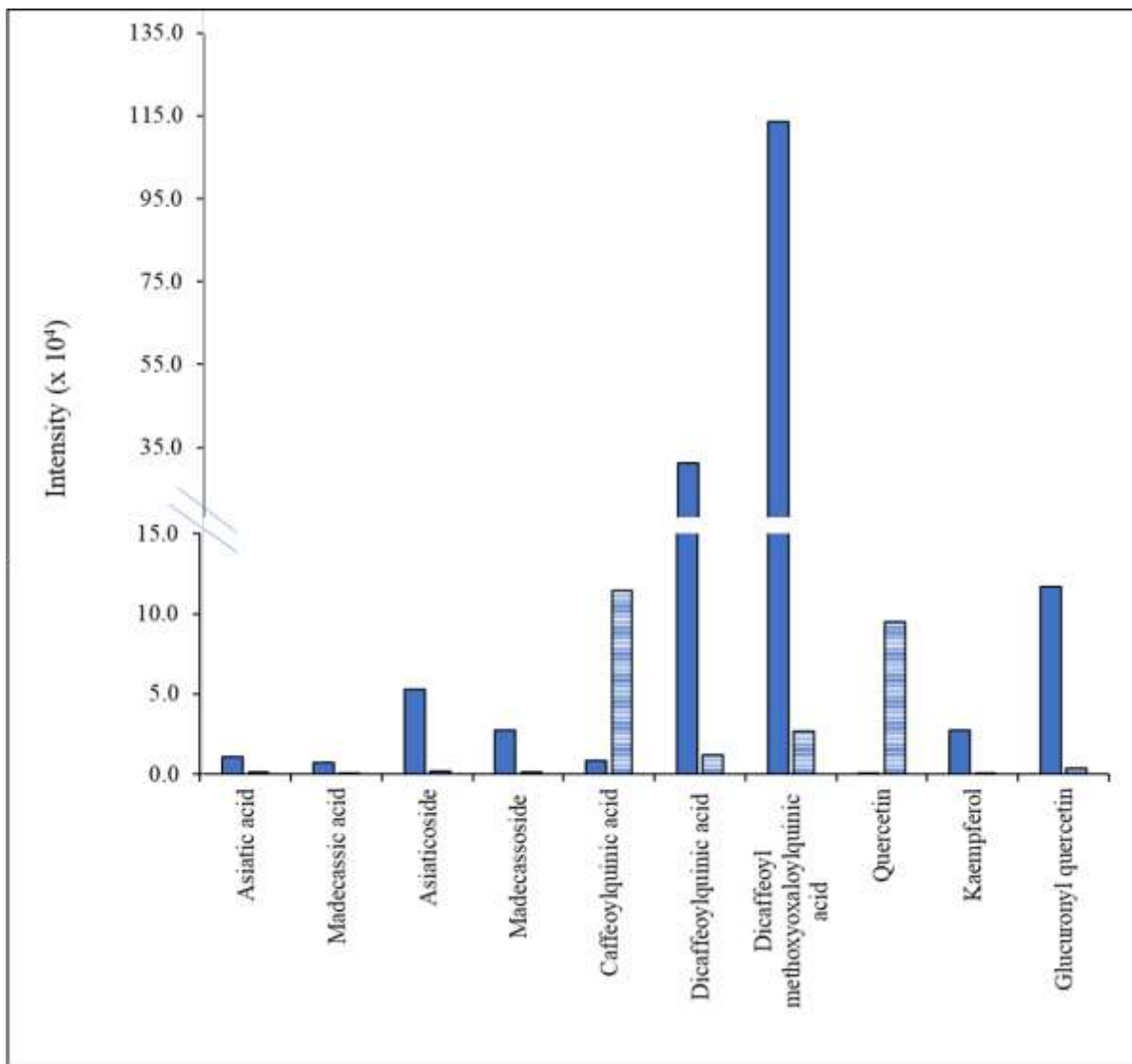
Future of Food Journal (FoFJ) Template

- 450 Nagoor Meeran, M.F., Goyal, S.N., Suchal, K., Sharma, C., Patil, C.R., & Ojha. S.K. (2018).
451 Pharmacological properties, molecular mechanisms, and pharmaceutical development
452 of asiatic acid: a pentacyclic triterpenoid of therapeutic promise. *Frontiers in*
453 *Pharmacology*, 9, 892. [doi:10.3389/fphar.2018.00892](https://doi.org/10.3389/fphar.2018.00892)
- 454 Navarajan, J., Jeyaramya, V., Oviya, R., Monica, A., & Anandhalakshmi, K. (2015). Plant
455 disease detection using Prewitt algorithm and neural network in image processing.
456 *International Journal of Electrical and Electronic Engineering & Telecommunications*,
457 1(1), 1-7.
- 458 Pękal, A., & Pyrzynska, K. (2014). Evaluation of aluminium complexation reaction for
459 flavonoid content assay. *Food Analytical Methods*, 7, 1776–1782. [doi:10.1007/s12161-](https://doi.org/10.1007/s12161-014-9814-x)
460 [014-9814-x](https://doi.org/10.1007/s12161-014-9814-x)
- 461 Plunkett, G.M., Chandler, G.T., Lowry, P.P., Pinney, S.M., & Sprenkle, T.S. (2004). Recent
462 advances in understanding Apiales and a revised classification. *South African Journal of*
463 *Botany*, 7(3), 371–381. [doi:10.1016/S0254-6299\(15\)30220-9](https://doi.org/10.1016/S0254-6299(15)30220-9)
- 464 Pulido, R., Bravo, L., & Saura-Calixto, F. (2000). Antioxidant activity of dietary
465 polyphenols as determined by a modified ferric reducing antioxidant power assay.
466 *Journal of Agricultural and Food Chemistry*, 48(8), 3396-3402. [doi:10.1021/jf9913458](https://doi.org/10.1021/jf9913458)
- 467 Sack, L., Dietrich, E.M., Streeter, C.M., Sánchez-Gómez, D., & Holbrook, N.M. (2008). Leaf
468 palmate venation and vascular redundancy confer tolerance of hydraulic disruption.
469 *Proceedings of the National Academy of Sciences of the United States of America*, 105,
470 1567–1572. [doi:10.1073/pnas.0709333105](https://doi.org/10.1073/pnas.0709333105)
- 471 Scoffoni, C., Rawls, M., Mckown, A., Cochard, H., & Sack, L. (2011). Decline of leaf
472 hydraulic conductance with dehydration: relationship to leaf size and venation
473 architecture. *Plant Physiology*, 156, 832–843. [doi:10.1104/pp.111.173856](https://doi.org/10.1104/pp.111.173856)
- 474 Siddiqui, N., Rauf, A., Latif, A., & Mahmood, Z. (2017). Spectrophotometric
475 determination of the total phenolic content, spectral and fluorescence study of the
476 herbal Unani drug Gul-e-Zoofa (*Nepeta bracteata* Benth). *Journal of Taibah University*
477 *Medical Sciences*, 12, 360–363.
- 478 Srivastava, R., Shukla, Y.N., & Kumar, S. (1997). Chemistry and pharmacology of *Centella*
479 *asiatica*: a review. *Journal of Medicinal and Aromatic Plants*, 19, 1049–1056
- 480 Strosnider, W.H.J., Winfrey, B.K., & Nairn, R.W. (2011). Acid mine drainage at Cerro Rico
481 de Potosí I: unabated high-strength discharges reflect a five century legacy of mining.
482 *Journal of Environmental Quality*, 40, 206–213.



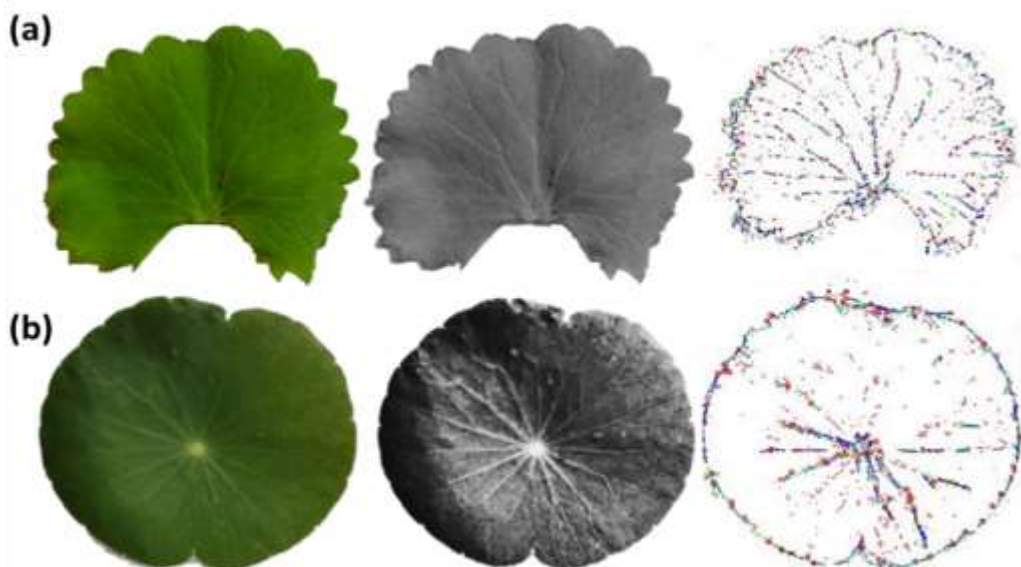
Future of Food Journal (FoFJ) Template

- 483 Subositi, D., Widodo, H., & Supriyati, H. (2016). Screening of ISSR markers for Pegagan
484 [*Centella asiatica* [L.] Urb] Authentication. *Buletin Plasma Nutfah*, 22(1), 49–54.
485 [doi:10.21082/blpn.v22n1.2016.p49-54](https://doi.org/10.21082/blpn.v22n1.2016.p49-54)
- 486 Sujanal, P., & Sankaran, K.V. (2016). The plants: Description, distribution, uses and
487 other information. In *Common plants of Maldives* (pp. 146). Bangkok, Thailand: Food
488 and Agriculture Organization of the United Nations, Kerala Forest Research Institute,
489 Kerala India.
- 490 Urban, I. (1879). *Centella asiatica* (L.) Urb. *Flora Bras.* 11, 287.
- 491 Vilasini, M., & Ramamoorthy, P. (2020). CNN approaches for classification of Indian leaf
492 species using smartphones. *Computers, Materials and Continua*, 62, 1445–1472.
- 493 Yang, R.Y., Lin, S., & Kuo, G. (2008). Content and distribution of flavonoids among 91
494 edible plant species. *Asia Pacific Journal of Clinical Nutrition*, 17(S1), 275-279.
- 495
- 496
- 497
- 498
- 499
- 500
- 501
- 502
- 503
- 504



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.



510

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

513

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

<i>Hydrocotyle verticillata</i>	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)	quercetin 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)

Future of Food Journal (FoFJ) Template

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

515

1 Comparing Reveal the herbal phytochemicals inof different Pegaga : 2 *Centella asiatica* and *Hydrocotyle verticillata*

5 **Abstract**

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

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

21 1.

27 **1- Introduction**

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

*Corresponding Author: Lee Suan Chua

Tel.: +6019-7214378; Email: chualeesuan@utm.my

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

46 This herb has been widely used as folk remedies for thousands of years (Diwan, et al., 1991).
47 Recent publication also supports the beneficial use of the herb through scientific studies.
48 Scientists and researchers are getting interested to generate technical data in line with the
49 traditional remedies. The ever-increasing use of the herb has caused the problem of
50 adulteration purposely or unintentionally with cheaper material. The common material that
51 has been mistreated is *Hydrocotyle bonariensis* Comm. ex Lam, which is usually called as
52 largeleaf pennywort or coast pennywort from the plant family Araliaceae (Plunkett, et al.,
53 2004). This exotic aquatic macrophyte is also called as Ulam Pegaga which means Pegaga
54 salad in Malaysia. Similar phenomenon is happening in Indonesia. The researchers reported
55 that *C. asiatica* is potentially adulterated with either *Hydrocotyle verticillata* or *Merremia*
56 *emarginata* which have same local name as Pegagan (Subositi, et al., 2016; Maruzy, et al.,
57 2020). The misidentification has also been happened in Philippines by local folks (Daminar
58 & Bajo, 2013). *H. bonariensis* is primarily planted in canals and water features for aesthetics
59 and phytoremediation (Strosnider, et al., 2011). The juice of the plant is traditionally
60 prepared to treat fever, colds, coughs, hepatitis, influenza, pruritus and sore throat, as well
61 as headaches and urinary problems (Sujanapal & Sankaran,
62 2016<http://florawww.eeb.uconn.edu/ipm.html>). [DC5][CL6] In 2014, a group of researchers
63 from Singapore compared the vegetative differences of *C. asiatica* and *H. verticillata*. *H.*
64 *verticillata* which is also known as water pennywort or whorled marsh-pennywort, is an
65 exotic aquatic macrophyte that is commonly found in marshes. The difference between both
66 species, in term of phytochemicals is extremely limited in literature. The difference of
67 phytochemicals in both species is of great importance, especially for herbal product
68 formulation.

69 Plant recognition is still the specialization of plant taxonomists and botanists with adequate
70 experience to authenticate plant species. The advancement of computing technologies and
71 invention of digital cameras have supported the works of non-specialists. The approach is
72 known as digital image processing which eases herbal identification in a rapid, simple, and
73 effective manner. The leaf features such as edge or shape, vein, dimension and colour appear
74 to be reliable inputs being considered in computing. Works have been extensively carried
75 out on leaf image processing and plant classification using different algorithms (Azlah, et al.,
76 2019). To the best of our knowledge, studies have not been performed to relate
77 phytochemicals and leaf morphological observation for plant recognition [DC7][CL8]. Most
78 probably, there are two different fields of studies in which cross disciplinary collaboration is
79 relatively limited in academia. Therefore, this study was carried out to investigate the

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

82 ~~2.~~ **2. Materials and Methods**

83 **2.1. —Phytochemical extraction**

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

91 **2.2. —Total Phenolic Content**

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

101 **2.3. —Total Flavonoid Content**

102 The total flavonoid content of samples was also estimated using the colorimetric method
103 (Aryal, et al., 2019). An aliquot of 1 mL sample was mixed with 3 mL methanolic AlCl₃
104 solution (10 %w/v), 0.2 mL potassium acetate (1 M) and 5.6 mL distilled water. The mixture
105 was incubated at 25 °C for 30 min and followed by the measurement of absorbance at 420
106 nm using a UV-Vis spectrophotometer. The results are expressed as milligram quercetin
107 equivalent per gram sample (mg QE/g).

108 **2.4. —Total triterpenoid content**

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

117 2.5. —Free radical scavenging activity

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

$$126 \text{ Inhibition (\%)} = \frac{A_o - A_s}{A_o} \times 100 \quad (1)$$

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

128 2.6. —Cation radical scavenging activity

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

138 2.7. —Reducing power

139 The reducing power of samples was determined using ferric reducing antioxidant power
140 (FRAP) assay which was carried out according to the procedures reported by [Chua et al.](#)
141 [\(2013\)](#) with modification. FRAP reagent was freshly prepared by mixing 2.5 mL 2,4,6-
142 tripyridyl-s-triazine complex (10 mM, Fe³⁺-TPTZ) in hydrochloric acid (40 mM), 2.5 mL iron
143 (III) chloride (20 mM, FeCl₃) and 25 mL acetate buffer (0.3 M, pH 3.6). The reagent solution
144 was kept in the dark at 37°C before use. Sample (0.2 mL) was mixed with 1.8 mL FRAP
145 reagent, and incubated at room temperature under subdued light condition for 10 min. The
146 absorbance was measured at 593 nm using a UV-Vis spectrophotometer. Ascorbic acid (10
147 mg/L) was used as standard chemical.

148 2.8. —Compound screening by LC-MS/MS

149 A Liquid chromatography (Ultimate 3000; Dionex Corporation; Sunnyvale, CA, USA)
150 integrated with a diode array detector (Dionex Ultimate 3000) and a tandem mass
151 spectrometer (QSTAR Elite; AB Sciex; Foster City, CA, USA) was used for compound
152 screening. Compounds were separated by a C18 XSelect HSS T3 column (2.1 mm × 100 mm,
153 2.5 µm) at a flow rate of 150 µL/min. A binary solvent system consisted of solvent A (water

154 with 0.1% formic acid) and solvent B (acetonitrile) was used as the mobile phase at the
155 following gradient: 0–10 min, 10% B; 10–20 min, 10–80% B; 20–25 min, 80% B; 25–25.1
156 min, 80–10% B; and 25.1–30 min, 10% B. The injection volume was 5 μ L. Compounds were
157 eluted from the column and detected at the wavelength of 254 nm. Subsequently, compounds
158 were ionized by a turbo ion spray (-4,500 V) before mass detection at the negative ion mode.
159 The mass range was set at the range of 100–1000 m/z. Nitrogen gas was used for curtain gas
160 (25 psi) and nebulizing gas (40 psi). The declustering potential was 40 V, whereas the
161 focusing potential was 200 V. Samples were filtered using a 0.2 μ m nylon membrane filter
162 prior to injection.

163 **2.9. —Leaf morphological recognition**

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

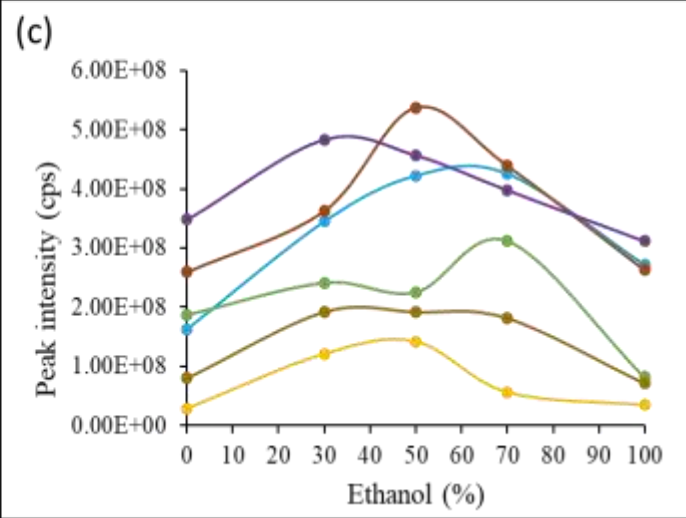
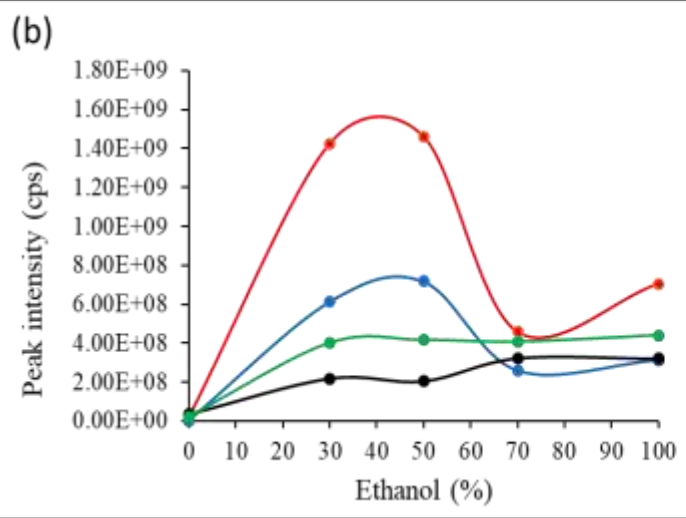
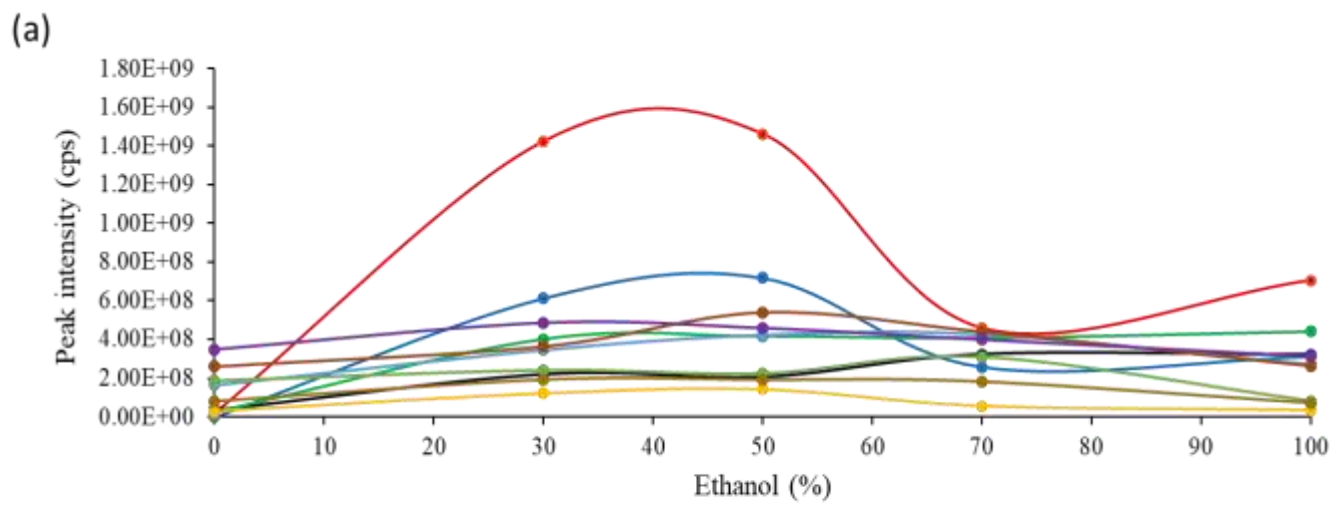
174 **2.10. —Multivariate data analysis**

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

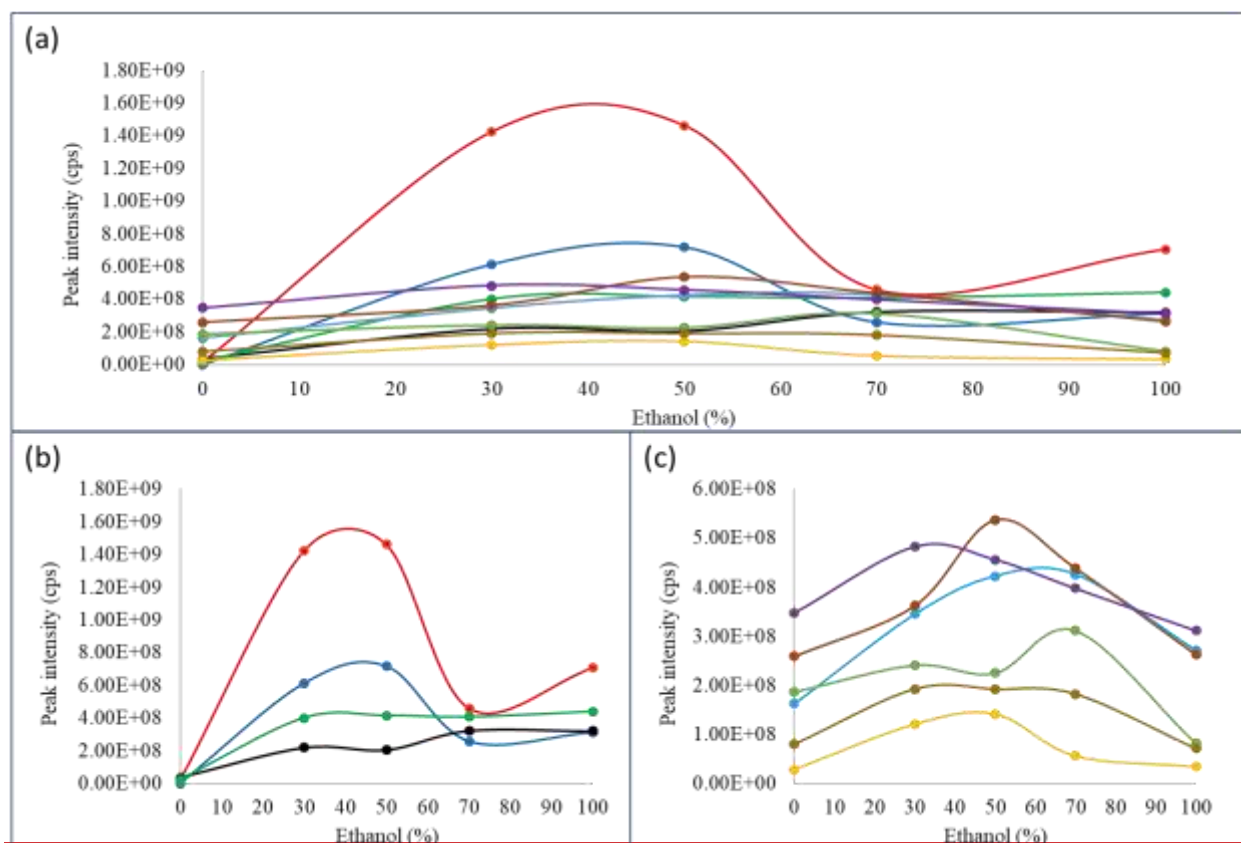
179 **3. —Results and Discussion**

180 **3.1. —High throughput mass screening**

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

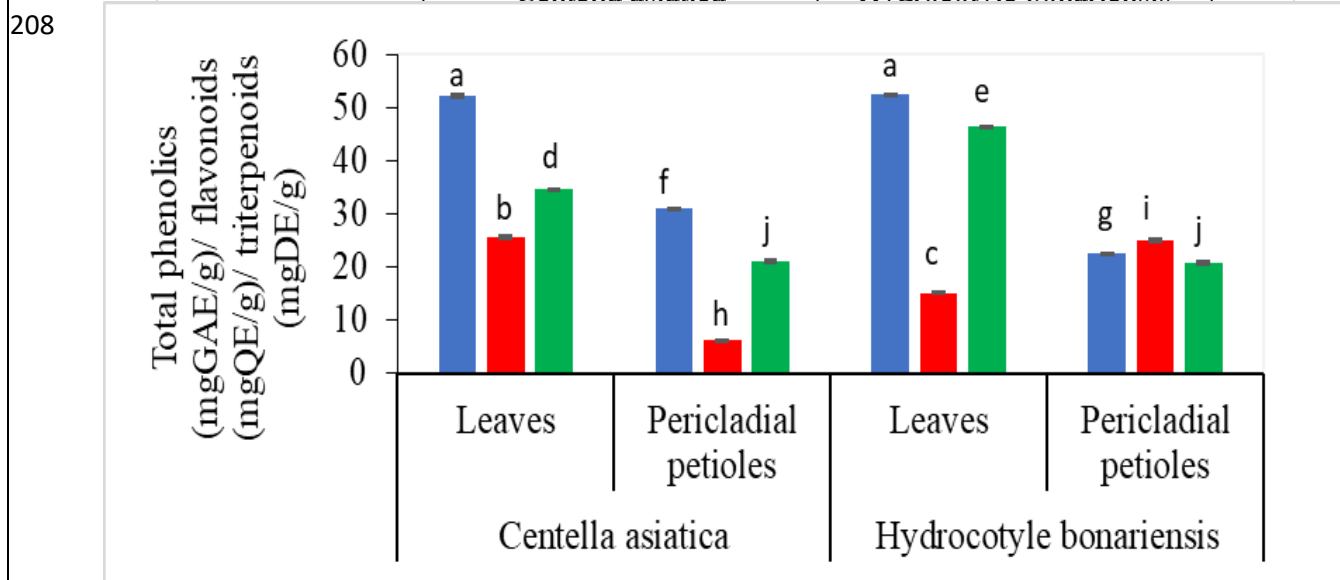
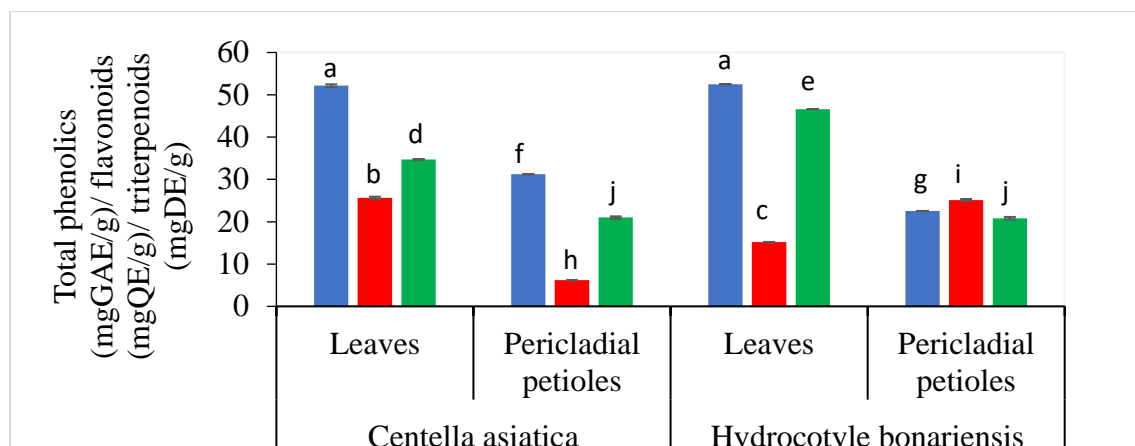


191



192
 193 Figure 1. (a) Ten target phytochemicals consisted of (b) two triterpenoids and their trisaccharides
 194 and (c) three phenolic acids, two flavonoids and one glycosylated flavonoid in the extracts of
 195 *Centella asiatica* prepared using different ethanol concentrations, where —●— asiatic acid, —●—
 196 madeassic acid, —●— asiaticoside, —●— madecassoside, —●— caffeoylquinic acid, —●— dicaffeoylquinic
 197 acid, —●— dicaffeoylmethoxyoxaloylquinic acid, —●— quercetin, —●— kaempferol and —●— glucuronyl
 198 quercetin

199
 200 In the subsequent analysis, 50% ethanolic extracts of the leaves and pericladial petioles of *C.*
 201 *asiatica* were examined for total phenolic, flavonoid and triterpenoid content
 202 spectrophotometrically (Figure 2). The results showed that leaf extract exhibited higher
 203 content of phytochemicals such as phenolics, flavonoids and triterpenoids than pericladial
 204 petiole extract. The proximate content of phytochemicals was also compared with its
 205 mimicking counterpart, *H. verticillata*. The comparison revealed that both herbal species had
 206 different compositions of phytochemicals, and phenolics was being the largest
 207 phytochemical group in the samples (Figure 2).



208

209

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

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

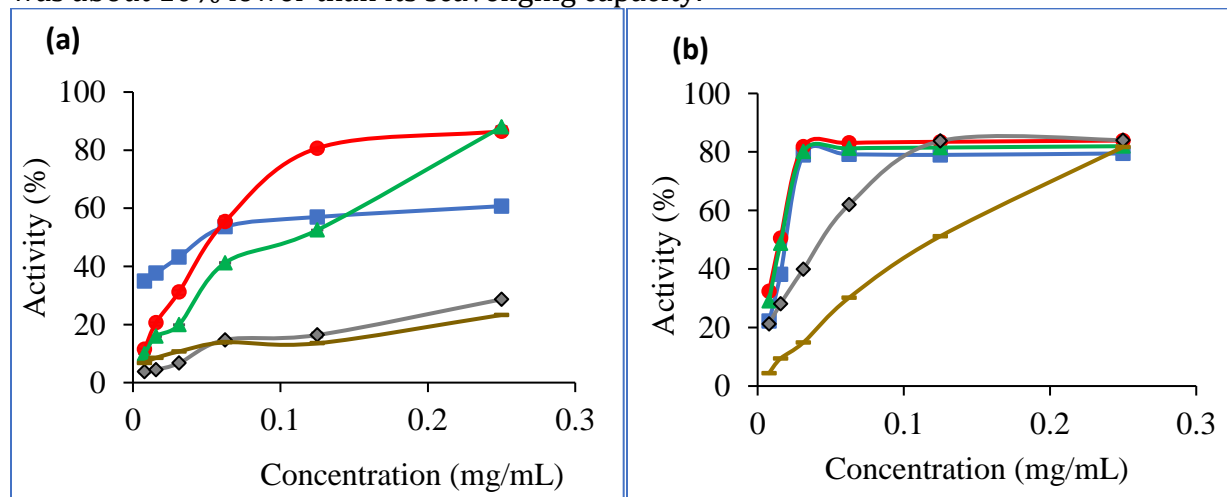
220

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

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

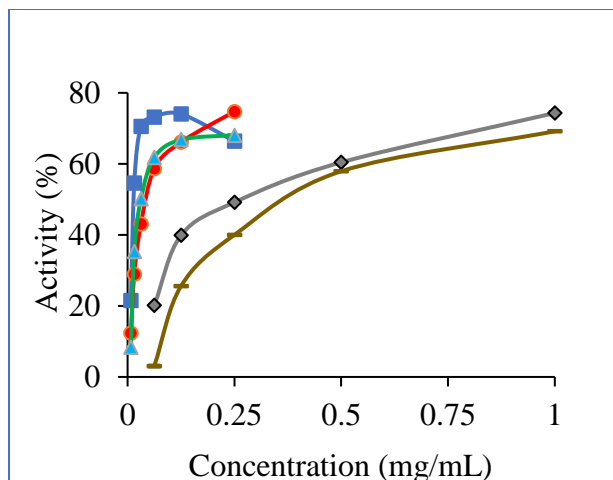
229 In the present study, quercetin was used as a standard chemical to build the calibration curve
230 of total flavonoid content. The absorbance was attributed to the formation of acid labile
231 complexes after chelating flavonoids with aluminum ions. Possibly, the C-4 keto, C-3 or C-5
232 hydroxyl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

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

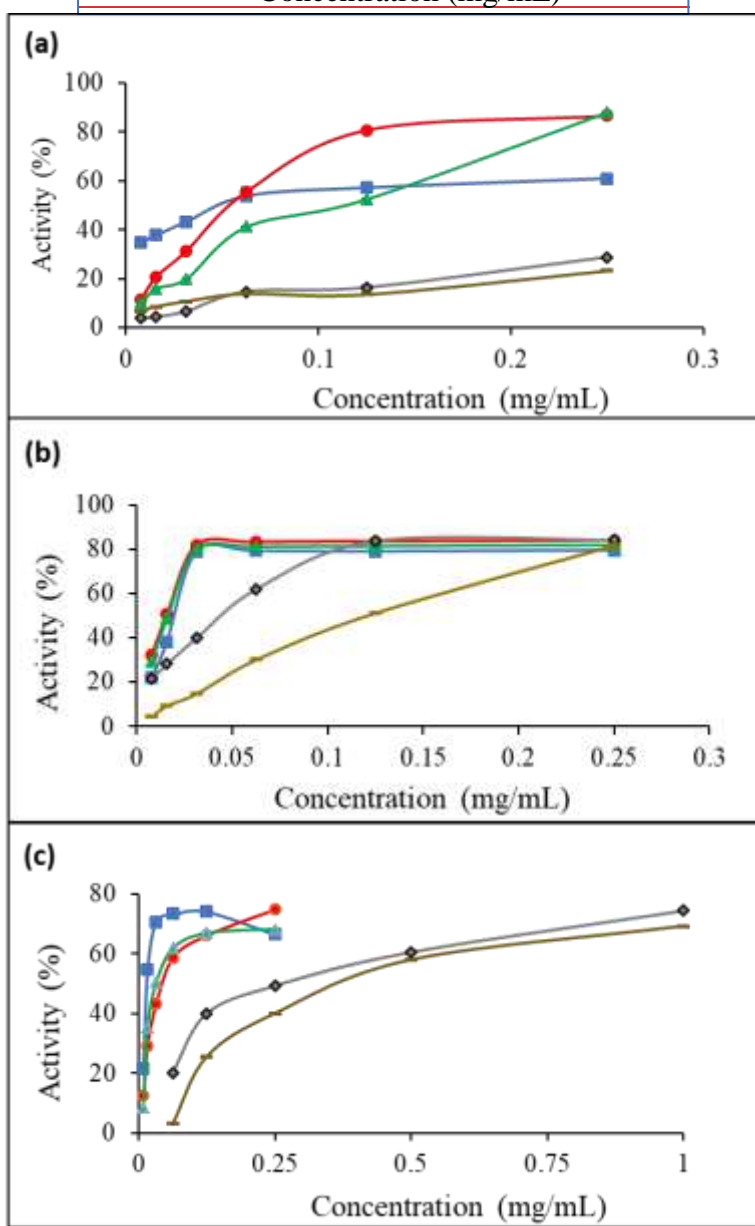


248

249



250

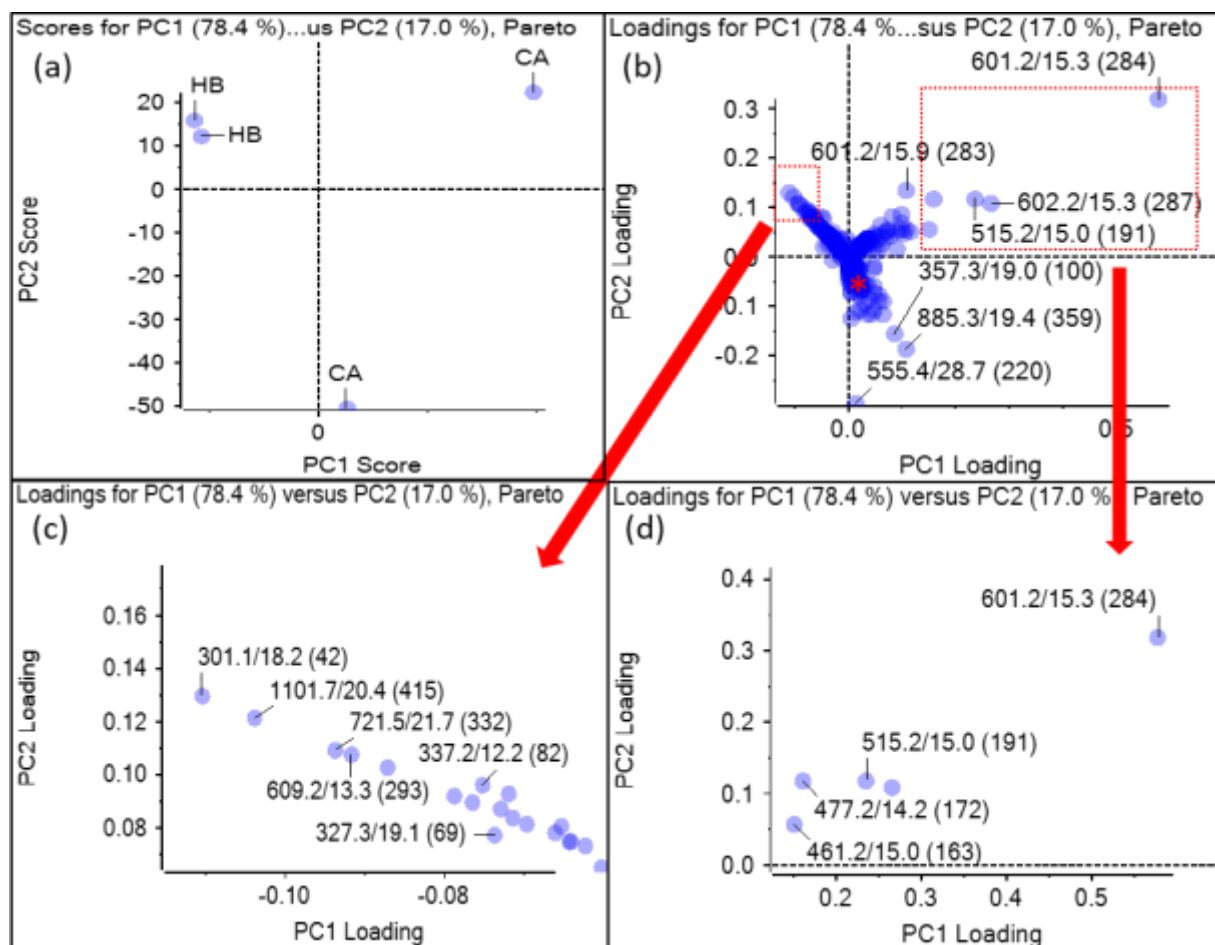


251 Figure 3. Antioxidant capacity of extracts based on the scavenging activities of (a) free and (b)
252 cation radicals, as well as (c) reducing power of ferric ions for ascorbic acid (■), the leaf extract
253 of *Centella asiatica* (●), the leaf extract of *Hydrocotyle verticillate* (▲), the pericladial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

255
256
257 The antioxidant compounds primarily follow the electron transfer mechanism to inhibit the
258 radicals. The compounds might also involve in hydrogen atom transfer at a slower rate
259 (Gulcin, 2020). Therefore, compounds with bulky rings would have the difficulty to access
260 radicals for electron transfer. On the other hand, compounds with conjugated double bonds
261 and multiple hydroxyl groups would be the dominant chemical characteristics to inhibit
262 radicals. DPPH assay is considered to be more selective because aromatic acid with a single
263 hydroxyl group does not react with DPPH radicals (Cerretani & Bendini, 2010). This also
264 indicates that the leaf extract of *C. asiatica* may have many polyol phenolics either from the
265 group of phenolic acids or polyphenols.

266 Compounds react with ABTS radicals would also respond to the FRAP assay because of
267 similarity in redox potentials (Gulcin, 2020). However, the results showed to have higher
268 concentration of samples to inhibit 50% of ferric ions. The lower reducing power could only
269 be contributed by water soluble antioxidative compounds (Apak, et al., 2007). The acidic
270 medium of FRAP assay was used to promote ferric ion solubility which indirectly increased
271 the redox potential. Pulido et al. (2000) reported that the absorbance of compounds such as
272 caffeic acid, quercetin and tannic acid was not stabilized even after several hours of reaction
273 time in FRAP assay. The observation was in good agreement with previous researchers that
274 antioxidant activity measured in FRAP assay was lower than that in ABTS assay (Gulcin,
275 2020).

276 The variance of phytochemicals in both herbs could be clustered into 3 major principal
277 components. The unsupervised multivariate analysis indicated that the phytochemicals in
278 both herbs could achieve up to 78.4 % of the total variance for the first principal component
279 (PC1). Figure 4 shows the phytochemicals in *C. asiatica* are prone to be located at the positive
280 region, whereas the phytochemicals in *H. hydrocotyle* are mostly located at the negative
281 region of PC1. The phytochemicals such as m/z 301 (quercetin), 353 (caffeoylquinic acid),
282 609 (glucosylrhamnosyl quercetin or rutin), 721 (tricafeoyl-2,7-anhydro-2-
283 octulopyranosonic acid) and 1101 (saponin) are likely to be the dominant compounds
284 differentiating *H. hydrocotyle* from *C. asiatica* (Figure 4(c)). Although m/z 461 (unknown),
285 477 (glucuronoyl quercetin), 515 (glycosyl caffeoylquinic acid) and 601 (dicafeoyl
286 methoxyoxaloylquinic acid) were found in both plant species, they were present in higher
287 amount in *C. asiatica* (Figure 4(d)). The pentacyclic triterpenoids and their trisaccharides
288 were located near the center of the axis as indicated in Figure 4.



289
 290 Figure 4. (a) Score and (b) loading plots of *Centella asiatica* (CA) and *Hydrocotyle verticillata*
 291 (HB) with the zoom-in area of masses, specifically for (c) HB in the negative region and (d) CA
 292 in the positive region of first principal component. * is the location of the pentacyclic triterpenoids
 293 and their trisaccharides in *C. asiatica*.

294

295 3.2. —Comparison of target phytochemicals

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

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

311

312 3.3. —Differentiation of leaf morphology

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

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

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

340 4. —Conclusion

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

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

348

349 **Acknowledgements**

350 This study was funded by Universiti Teknologi Malaysia (TDR-07G21-06G75 and HR-08G84).

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.

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

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

354

355 **References**

356 Apak, R., Güçlü, K., Demirata, B., Ozyürek, M., Celik, S.E., Bektaşoğlu, B., Berker, K.I., & Ozyurt,
357 D. (2007). Comparative evaluation of various total antioxidant capacity assays applied to
358 phenolic compounds with the CUPRAC assay. *Molecules*, 12(7), 1496-1547.
359 <https://doi.org/doi:10.3390/12071496>

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

363 Azlah, M.A.F., Chua, L.S., Rahmad, F.R., Abdullah, F.I., & Wan Alwi, S.R. (2019). Review on
364 Techniques for Plant Leaf Classification and Recognition. *Computers*, 8, 77.
365 <https://doi.org/doi:10.3390/computers8040077>

366 Biskup, I., Golonka, I., Gamian, A., & Sroka, Z. (2013). Antioxidant activity of selected phenols
367 estimated by ABTS and FRAP methods. *Postępy Higieny i Medycyny Doświadczalnej, Postępy*
368 *Hig Med Dosw* 67, 958-963. <http://www.phmd.pl/fulltxt.php?ICID=1066062>

369 Brinkhaus, B., Lindner, M., Schuppan, D., & Hahn, E.G. (2000). Chemical, pharmacological and
370 clinical profile of the East Asian medical plant *Centella asiatica*. *Phytomedicine*, 7, 427-448.
371 [https://doi.org/doi:10.1016/S0944-7113\(00\)80065-3](https://doi.org/doi:10.1016/S0944-7113(00)80065-3)

372 Brito, A., Ramirez, J.E., Areche, C., Sepúlveda, B., & Simirgiotis, M.J. (2014). HPLC-UV-MS
373 profiles of phenolic compounds and antioxidant activity of fruits from three citrus species
374 consumed in Northern Chile. *Molecules* [DC13][CL14], 19, 17400-17421.
375 <https://doi.org/doi:10.3390/molecules191117400>

376 Cerretani, L., & Bendini, A. (2010). Rapid assays to evaluate the antioxidant capacity of
377 phenols in virgin olive oil. ~~In: Olives and Olive Oil in Health and Disease Prevention~~, pp-625-
378 635. [doi:10.1016/B978-0-12-374420-3.00067-X](https://doi.org/doi:10.1016/B978-0-12-374420-3.00067-X)

379 Chaisawang, P., Sirichoat, A., Chaijaroonkhanarak, W., Pannangrong, W., Sripanidkulchai, B.,
380 Wigmore, P., & Welbat, J.U. (2017). Asiatic acid protects against cognitive deficits and
381 reductions in cell proliferation and survival in the rat hippocampus caused by 5-fluorouracil
382 chemotherapy. *PLoS ONE*, 12(7), e0180650.
383 <https://doi.org/doi:10.1371/journal.pone.0180650>

384 Chen, G., Li, X., Saleri, F., & Guo, M. (2016). Analysis of flavonoids in *Rhamnus davurica* and its
385 antiproliferative activities. *Molecules*, 21, 1275.
386 <https://doi.org/doi:10.3390/molecules21101275>

387 Chu, Y., Chang, C., & Hsu, H. (2000). Flavonoid content of several vegetables and their
388 antioxidant activity. *Journal of the Science of Food and Agriculture*, 80, 561-566.
389 [https://doi.org/doi:10.1002/\(SICI\)1097-0010\(200004\)80:5<561::AID-](https://doi.org/doi:10.1002/(SICI)1097-0010(200004)80:5<561::AID-JSFA574>3.0.CO;2-%23)
390 [JSFA574>3.0.CO;2-%23](https://doi.org/doi:10.1002/(SICI)1097-0010(200004)80:5<561::AID-JSFA574>3.0.CO;2-%23)

391 Chua, L.S., Lau, C.H., Chew, C.Y., & Dawood, D.A.S, (2019). Solvent fractionation and acetone
392 precipitation for crude saponins from *Eurycoma longifolia* extract. *Molecules*, 24, 1416.
393 <https://doi.org/doi:10.3390/molecules24071416>

394 Chua, L.S., Rahaman, N.L.A., Adnan, N.A., & Tan, T.T.E. (2013). Antioxidant activity of three
395 honey samples in relation with their biochemical components. *Journal of Analytical Methods*
396 *in Chemistry*, 2013, 1-8. <https://doi.org/doi:10.1155/2013/313798>

397 Daminar, N.L., & Bajo, L.M. (2013). Isolation and partial characterization of the most
398 bioactive metabolite from the hexane extract of the aerial part of *Hydrocotyle verticillata*
399 (whorled marshpennyworth). *Global Journal of Science Frontier Research*, 13(2), 1-8.

400 Diwan, P.C., Karwande, I., & Singh, A.K. (1991). Anti-anxiety profile of mandukparni (*Centella*
401 *asiatica*) Linn in animals. *Fitoterapia*, 62, 255-257.

402 Ecology & Evolutionary Biology, Plant Biodiversity Conservatory and Research Core, USA,
403 Retrieved from <http://florawww.eeb.uconn.edu/ipm.html> (Accessed 26 June 2020).

404 Ehsani Rad, A. (2010). Plant Classification Based on Leaf Recognition. *International Journal*
405 *of Information Security*, 8, 77-81.[DC15][CL16]

406 Gohil, K.J., Patel, J.A., & Gajjar, A.K. (2010). Pharmacological review on *Centella asiatica*: a
407 potential herbal cure-all. *Indian Journal of Pharmaceutical Sciences*, 72, 546-556.

408 <https://doi.org/doi:10.4103/0250-474X.78519>

409 Gulcin, İ. (2020). Antioxidants and antioxidant methods: an updated overview. *Archives of*
410 *Toxicology*, 94, 651–715. <https://doi.org/doi:10.1007/s00204-020-02689-3>

411 Huang, S.S., Huang, G.J., Ho, Y.L., Lin, Y.H., Hung, H.J., Chang, T.N., Chang, M.J., Chen, J.J., &
412 Chang, Y.S. (2008). Antioxidant and antiproliferative activities of the four *Hydrocotyle* species
413 from Taiwan. *Botanical Studies*, 49(4), 311-322.

414 Jahan, R., Hossain, S., Seraj, S., Nasrin, D., Khatun, Z., Das, P.R., Islam, M.T., Ahmed, I., &
415 Rahmatullah, M. (2012). *Centella asiatica* (L.) Urb.: Ethnomedicinal uses and their scientific
416 validations. *American-Eurasian Journal of Sustainable Agriculture*, 6(4), 261-270.

417 Jamil, S.S., Nizami, Q., & Salam, M. (2007). *Centella asiatica* (Linn.) Urban: a review. *Natural*
418 *Product Radiance*, 6(2), 158–170. <http://nopr.niscair.res.in/handle/123456789/7855>

419 Kasprzak, M.M., Erxleben, A., & Ochocki, J. (2015). Properties and applications of flavonoid
420 metal complexes. *Royal Society of Chemistry Advances*, 5, 45853-45877.
421 <https://doi.org/doi:10.1039/C5RA05069C>

422 Kavitha, C.V., Agarwal, C., Agarwal, R., & Deep G. (2011). Asiatic acid inhibits pro-angiogenic
423 effects of VEGF and human gliomas in endothelial cell culture models. *PLoS ONE*, 6(8),
424 e22745. <https://doi.org/doi:10.1371/journal.pone.0022745>

425 Lee, K.B., & Hong, K.S. (2013). An implementation of leaf recognition system using leaf vein
426 and shape. *International Journal of Bio-Science and Bio-Technology*, 5, 57–65.

427 Li, Z.H., Guo, H., Xu, W.B., Ge, J., Li, X., Alimu, M., & He, D.J. (2016). Rapid identification of
428 flavonoid constituents directly from PTP1B inhibitive extract of Raspberry (*Rubus idaeus* L.)
429 leaves by HPLC-ESI-QTOF-MS-MS. *Journal of Chromatographic Science*, 54(5), 05-810.
430 <https://doi.org/doi:10.1093/chromsci/bmw016>

431 Mangas, S., Moyano, E., Osuna, L., Cusido, R.M., Bonfill, M., & Palazo, J. (2008). Triterpenoid
432 saponin content and the expression level of some related genes in calli of *Centella asiatica*.
433 *Biotechnology Letters*, 30, 1853-1859. <https://doi.org/doi:10.1007/s10529-008-9766-6>

434 Maruzy, A., Budiarti, M., & Subositi, D. (2020). Authentication of *Centella asiatica* (L.) Urb.
435 (Pegagan) and its adulterant based on macroscopic, microscopic, and chemical profiling.
436 *Jurnal Kefarmasian Indonesia*, 10(1), 19-30. <https://doi.org/doi:10.22435/jki.v10i1.1830>

437 Maulidiani, Abas, F., Khatib, A., Shaari, K., & Lajis, N.H. (2014). Chemical characterization and
438 antioxidant activity of three medicinal Apiaceae species. *Industrial Crops and Products*, 55,
439 238–247. <https://doi.org/doi:10.1016/j.indcrop.2014.02.013>

440 Nagoor Meeran, M.F., Goyal, S.N., Suchal, K., Sharma, C., Patil, C.R., & Ojha, S.K. (2018).
441 Pharmacological properties, molecular mechanisms, and pharmaceutical development of

442 asiatic acid: a pentacyclic triterpenoid of therapeutic promise. *Frontiers in Pharmacology*, 9,
443 892. <https://doi.org/doi:10.3389/fphar.2018.00892>

444 Navarajan, J., Jeyaramya, V., Oviya, R., Monica, A., & Anandhalakshmi, K. (2015). Plant disease
445 detection using Prewitt algorithm and neural network in image processing. *International*
446 *Journal of Electrical and Electronic Engineering & Telecommunications*, 1(1), 1-7.

447 Pękal, A., & Pyrzynska, K. (2014). Evaluation of aluminium complexation reaction for
448 flavonoid content assay. *Food Analytical Methods*, 7, 1776–1782.
449 <https://doi.org/doi:10.1007/s12161-014-9814-x>

450 Plunkett, G.M., Chandler, G.T., Lowry, P.P., Pinney, S.M., & Sprengle, T.S. (2004). Recent
451 advances in understanding Apiales and a revised classification. *South African Journal of*
452 *Botany*, 7(3), 371–381. [https://doi.org/doi:10.1016/S0254-6299\(15\)30220-9](https://doi.org/doi:10.1016/S0254-6299(15)30220-9)

453 Pulido, R., Bravo, L., & Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as
454 determined by a modified ferric reducing antioxidant power assay. *Journal of Agricultural*
455 *and Food Chemistry*, 48(8), 3396–3402. <https://doi.org/doi:10.1021/jf9913458>

456 Sack, L., Dietrich, E.M., Streeter, C.M., Sánchez-Gómez, D., & Holbrook, N.M. (2008). Leaf
457 palmate venation and vascular redundancy confer tolerance of hydraulic disruption.
458 *Proceedings of the National Academy of Sciences of the United States of America*, 105, 1567–
459 1572. <https://doi.org/doi:10.1073/pnas.0709333105>

460 Scoffoni, C., Rawls, M., Mckown, A., Cochard, H., & Sack, L. (2011). Decline of leaf hydraulic
461 conductance with dehydration: relationship to leaf size and venation architecture. *Plant*
462 *Physiology*, 156, 832–843. <https://doi.org/doi:10.1104/pp.111.173856>

463 Siddiqui, N., Rauf, A., Latif, A., & Mahmood, Z. (2017). Spectrophotometric determination of
464 the total phenolic content, spectral and fluorescence study of the herbal Unani drug Gul-e-
465 Zoofa (*Nepeta bracteata* Benth). *Journal of Taibah University Medical Sciences*, 12, 360–363.

466 Srivastava, R., Shukla, Y.N., & Kumar, S. (1997). Chemistry and pharmacology of *Centella*
467 *asiatica*: a review. *Journal of Medicinal and Aromatic Plants*, 19, 1049–1056

468 Strosnider, W.H.J., Winfrey, B.K., & Nairn, R.W. (2011). Acid mine drainage at Cerro Rico de
469 Potosí I: unabated high-strength discharges reflect a five century legacy of mining. *Journal of*
470 *Environmental Quality*, 40, 206–213.

471 Subositi, D., Widodo, H., & Supriyati, H. (2016). Screening of ISSR markers for Pegagan
472 [*Centella asiatica* [L.] Urb] Authentication. *Buletin Plasma Nutfah*, 22(1), 49–54.
473 <https://doi.org/doi:10.21082/blpn.v22n1.2016.p49-54>

474 [Sujanapal, P., & Sankaran, K.V. \(2016\). The plants: Description, distribution, uses and other](#)
475 [information. In Common plants of Maldives \(pp. 146\). Bangkok, Thailand: Food and](#)
476 [Agriculture Organization of the United Nations, Kerala Forest Research Institute, Kerala](#)
477 [India.](#)

478 Urban, I. (1879). *Centella asiatica* (L.) Urb. *Flora Bras.* 11, 287.

479 Vilasini, M., & Ramamoorthy, P. (2020). CNN approaches for classification of Indian leaf
480 species using smartphones. *Computers, Materials and Continua*, 62, 1445–1472.

481 Yang, R.Y., Lin, S., & Kuo, G. (2008). Content and distribution of flavonoids among 91 edible
482 plant species. *Asia Pacific Journal of Clinical Nutrition*, 17(S1), 275-279.

483

484

485

486

487

488

489

490

491

492

493

494

495

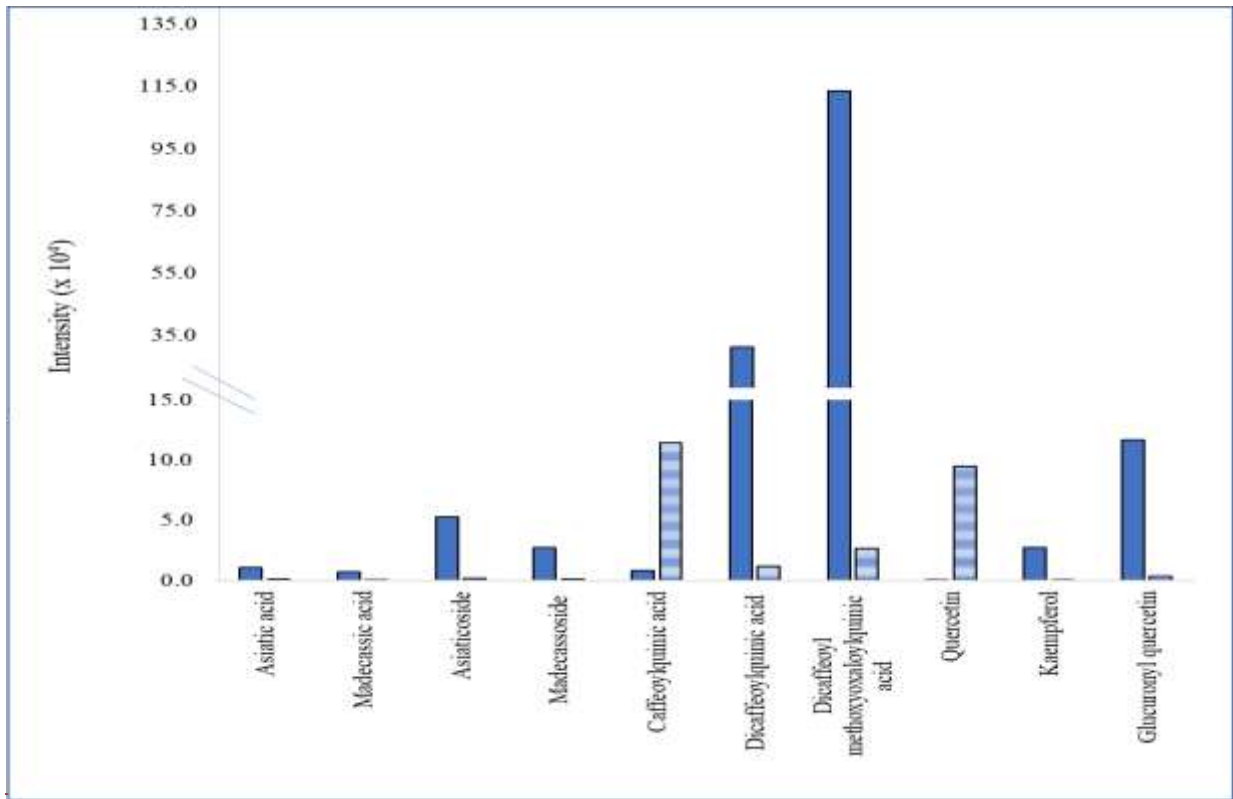
496

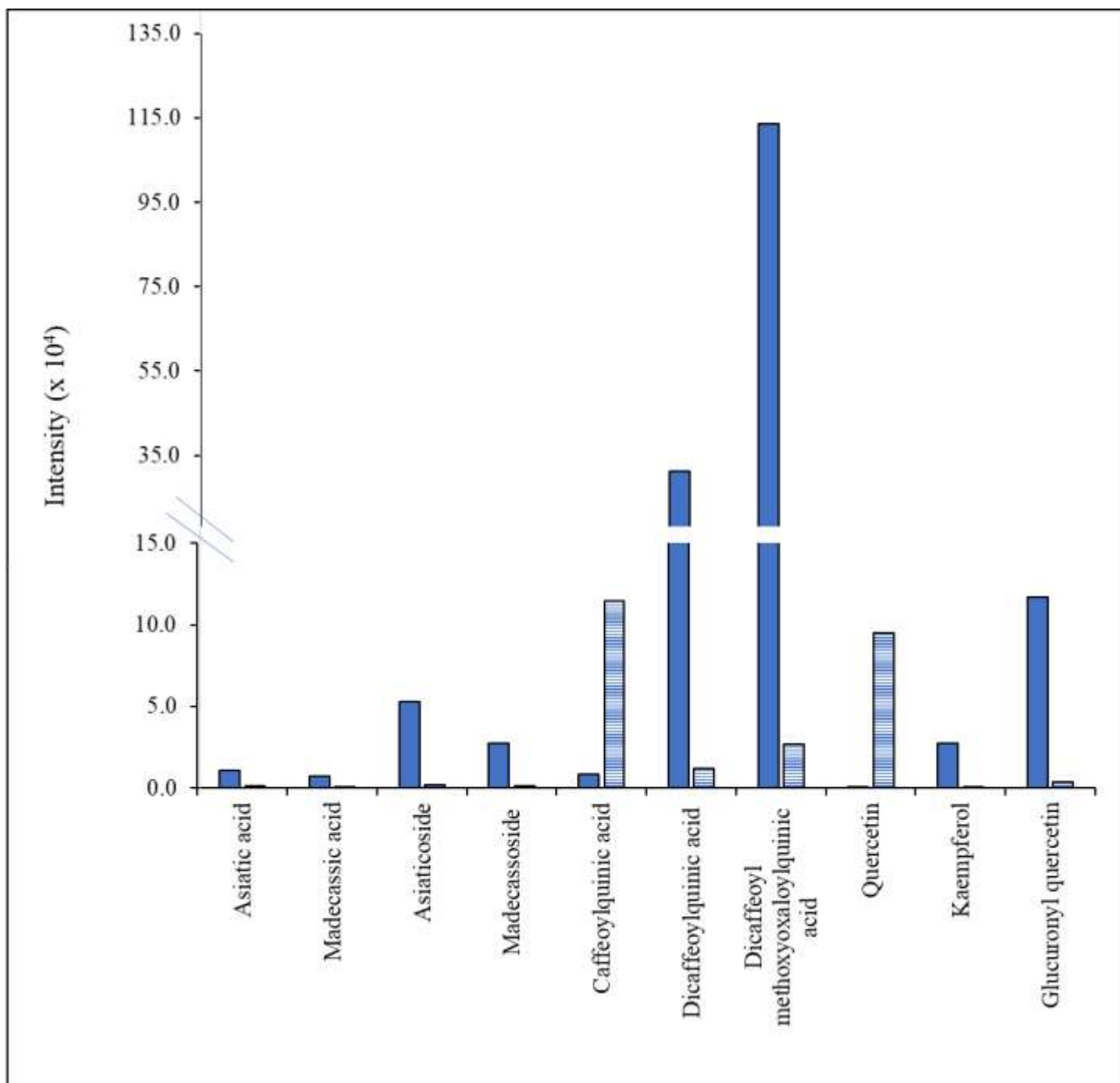
497

498

499 Supplementary

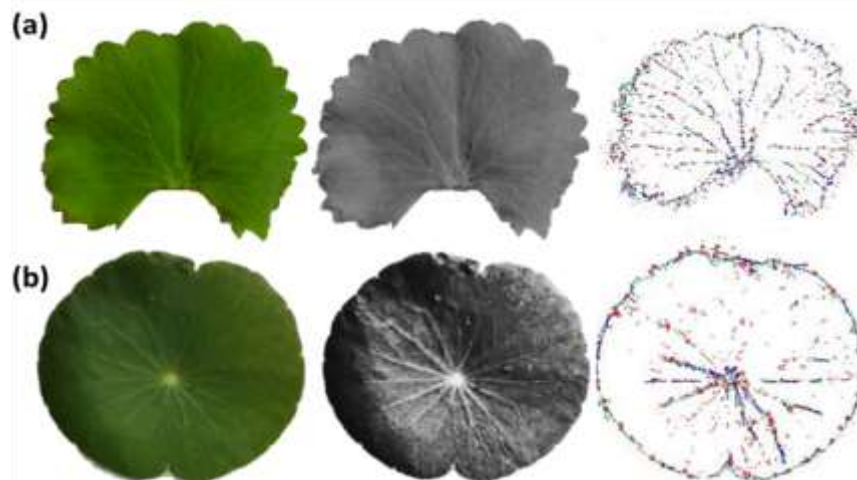
500





501

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



504
505 Figure 6 (supplementary). Original leaf morphological images of (a) *Centella asiatica* and (b)
506 *Hydrocotyle verticillata* which are converted to gray scale and pixel images

507
508 Table 1 (supplementary). Phytochemicals detected in *Hydrocotyle verticillata* extract

<i>Hydrocotyle verticillata</i>	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)	quercetin 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)

| 510
511