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MORPHOLOGICAL TRAITS, DNA BARCODING AND METABOLITE PROFILING FOR THE DISCRIMINATION OF TWO PEGAGA SPECIES

Woon Ling Chia[1], Mei Wei Lai[1], Yee Soon Ling[2], Yen Min Lan[3], Sheh May Tam[4], Chung Cheng Richard Kong[5], Ming Hock Haw[6] and Yang Mooi Lim[1,3*]

[1]*Centre for Cancer Research, Faculty of Medicine and Health Sciences, University of Tunku Abdul Rahman, Jalan Sungai Long, Bandar Sungai Long, Kajang, 43000, Selangor, Malaysia*
[2]*Biotechnology Research Institute, University Malaysia Sabah, Jalan UMS, 88400, Kota Kinabalu, 88400, Sabah, Malaysia*
[3]*Department of Preclinical Sciences, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, Jalan Sungai Long, Bandar Sungai Long, Kajang, 43000, Selangor, Malaysia*
[4]*School of Bioscience, Taylor University, Lakeside Campus, No 1, Jalan Taylor's, Subang Jaya, 47500, Selangor, Malaysia*
[5]*Forest Biodiversity Division, Forest Research Institute Malaysia, Jalan FRIM, Kepong, 52109, Kuala Lumpur, Malaysia*
[6] *KL Academy of Traditional Chinese Medicine, 138-140, Jalan Petaling, City Centre, 50000, Kuala Lumpur, Malaysia*

*Corresponding Author: ymlim@utar.edu.my

History

Keywords:

Centella asiatica; Core barcode; Hydrocotyle sibthorpioides; Medicinal Plant; Species discrimination

Abstract

In Malaysia, *Centella asiatica* and *Hydrocotyle sibthorpioides* are used interchangeably under the name of 'Pegaga'. This study aims to differentiate these two Pegaga species based on morphology traits, DNA barcoding, and metabolite profiles. These two species of Pegaga were collected from Negeri Sembilan and Johor, Malaysia. Their morphological characteristics, DNA barcodes and metabolite contents were investigated. Three DNA barcoding regions of ribuloase-1,5-biphosphate carboxylase oxygenase large subunit (*rbc*L), maturase K (*mat*K) and Internal Transcribed Spacer (ITS) were used to determine the DNA barcodes of these two Pegaga. Untargeted metabolite profiling of Pegaga extracts were determined using LC-MS/MS. The morphological characteristics of the two Pegaga species were distinct upon close investigation. Based on the morphological traits, LYMOOI 046 and LYMOOI 069 were identified as *C. asiatica* and *H. sibthorpioides*, respectively. Three DNA barcode regions for PCR and sequencing success rate were 100%. The results obtained from BLAST search suggest that the sequences of the plastid *rbc*L and *mat*K gene regions show consistency with the identity of Pegaga species as defined by morphological traits. However, ITS gene region is less effective for species identification as it unable to differentiate the Pegaga species. Metabolite profiling revealed that both the Pegaga species is chemically different, in which *C. asiatica* is high in both terpenoid and phenolic compounds, and *H. sibthorpioides* is high in phenolic compounds especially flavonoid glycosides. This work suggests that morphological traits, DNA barcoding and metabolite profiling can discriminate these two Pegaga species.

INTRODUCTION

Medicinal plants have slowly become the mainstream of nature source for modern drugs, this is due to the belief that natural remedies are more reliable and effective as compared to conventional drugs [1]. Pegaga is one of the medicinal plants found in Malaysia and has been widely used for generations as herbal medicine in the country. *Centella asiatica* and *Hydrocotyle sibthorpioides* are often used interchangeably as Pegaga for herbal treatment.

C. asiatica is considered as an ancient herb that plays a vast role in Indian system medicine, and traditional Chinese medicine as well as in folk remedies of Asia countries [2]. In Malaysia, it has been consumed as a dietary medicine for the purpose of treating hypertension and tonify the body [3]. Various pharmacological activities have been found in *C. asiatica* such as anti-inflammatory [4], antimicrobial [5], neuroprotective [6], and antioxidant properties [7]. *H. sibthorpioides* is a well-known folkloric used medicinal plant in the treatment of lymphadenitis, herpes zoster, acute hepatitis, and cholecystitis [8]. In India, the whole plant is used for treating malaria and associated symptoms [9]. A number of studies have reported antiviral properties of *H. sibthorpioides* against dengue virus [10] and hepatitis B virus [11].

Despite their popular use, uncertainties still exist between the Pegaga species, resulting in problems of standardization of raw materials for further downstream application. Pegaga is traded as products with local names in folk taxonomies. This leads to the issues of misidentification since vernacular of local names are prone to ambiguity as one name may apply to many divergent species or conversely one species can have multiple local names [12]. Although these two medicinal herbs showed some different morphological characteristics, they are still hardly distinguished morphologically by those without knowledge on taxonomy experiences. The limitation of morphological characteristics can be overcome by DNA barcoding. DNA barcoding is a technique which used a species-specific region of DNA to identify species [13]. For plant identification, Plant Working Group of the Consortium for the Barcode of Life (CBOL) in year 2009 suggested the use of ribuloase-1,5-biphosphate carboxylase oxygenase large subunit (*rbc*L) and maturase K (*mat*K) as standard barcode [14], meanwhile Internal Transcribed Spacer (ITS) were added as supplement [15].

Since there are many reports suggesting the utility of both Pegaga for various diseases, it is important to investigate the metabolite compounds of them. Metabolite profiling based on liquid chromatography coupled with mass spectrometry enables systematic and comprehensive investigation of metabolites of plant samples [16]. It offers greater speed and sensitive analyses [17]. Instead of using targeted analysis, untargeted metabolite profiling was chosen as it is able to measure a wider range of metabolite without prior information or metabolome knowledge of tested samples [18].

Accurate identification of Pegaga species is fundamental steps for the effective use of this herb. Morphological characterization, DNA study and metabolite contents play vital roles in the accurate identification and quality assessment of medical plants, and impact on respective pharmaceutical applications. The integration of these methods has successfully applied in differentiating numerous medicinal plants [19–21]. Hence, the objective of the present study was to distinguish the Pegaga species using morphological characteristics and DNA barcoding, as well as to investigate the metabolite profiles of Pegaga species.

MATERIALS AND METHODS

Plant Material

Collection of plant samples was carried out at Broga, Negeri Sembilan and Batu Pahat, Johor Malaysia, respectively. The geographical coordinates of the plant sample collection were N02°55'40.0" E101°55'40.6" for LYMOOI 046 and N1°58'54.1" E102°56'21.8" for LYMOOI 069. Photograph and herbarium samples of both the plants were taken in situ. One fresh sample per each studied plant were individually collected in different sterile plastic bags for the purpose of DNA isolation and LC-MS/MS extraction.

Morphological Identification

Both plants were identified by herbalist in the field. The identification was focused on the morphology of leaf shape, leaf margin, leaf size and plant height. The criteria of plant's height, leaves size was measured using measuring tape.

Herbarium and Taxonomic Identification

A voucher specimen number was assigned to each of the Pegaga. Data of each plant were recorded in accordance with a template of Forest Research Institute Malaysia. The herbarium specimens were verified and deposited at Perdana Botanical Garden Kuala Lumpur. The taxonomy data collection was recorded in the database BRAHMS (Botanical Research and Herbarium Management System). The herbarium voucher numbers are LYMOOI 046 and LYMOOI 069, respectively.

DNA Barcoding

DNA Isolation and PCR Amplification

To perform DNA extraction, whole plants were immersed in liquid nitrogen and were crushed using mortar and pestle to obtain fine powder. DNA extraction of the samples was performed using GeneJET Plant Genomic DNA Purification Kit (Thermo Fisher Scientific) following the manufacturer protocol. The extracted DNA was kept at -20°C for polymerase chain reaction (PCR). PCR was performed using Veriti 96 Well Thermal Cycle (Applied Biosystems). The three barcode loci for the coding gene *rbc*L, *mat*K and ITS primers (recorded in Table 1) were amplified using Phire Plant Direct PCR Mastermix (Thermo Scientific). The PCR products obtained were visualized on 1.5 % agarose gel. DNA ladder of DM2100 ExcelBand 100bp (SMOBIO) was used to estimate the size of amplification products. Band of the expected size was excised prior to being sent for DNA sequencing at Apical Scientific Malaysia.

Table 1: Details of primers and their thermocycling conditions used in the study.

Region	Primer	Sequence (5'-3')	Thermocycling condition	Reference
*rbc*L	rbcLaF	ATG TCA CCA CAA ACA GAG ACT AAA GC	94°C for 4 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; final extension 72°C for 10 min.	[74]
	rbcLaj634R	GAA ACG GTC TCT CCA ACG CAT		
*mat*K	matK_390f	CGA TCT ATT CAT TCA ATA TTT C	94°C for 3 min, 35 cycles of 94°C for 30 s, 48°C for 40 s, 72°C for 1 min, and a final extension at 72°C for 10 min.	[74]
	matK_1326r	TCT AGC ACA CGA AAG TCG AAG T		
ITS	ITS_5P	GGA AGG AGA AGT CGT AAC AAG G	94°C for 5 min, 35 cycles of 94°C for 30 s, 55°C for 40 s, 72°C for 1 min, and a final extension at 72°C for 10 min.	[75]
	ITS_8P	CAC GCT TCT CCA GAC TAC A		

DNA Sequence Alignment and Analysis

DNA sequencing was performed using the same primer used in DNA amplification. Bidirectional DNA sequence trace files were aligned separately with the program Mega 7.0. All the consensus nucleotides were queried using the default setting of megablast online at National Centre for Biotechnology Information (NCBI) against the nucleotide database. Identification assigned based on the top hit with identity more than 99%.

Untargeted Metabolite Profiling

Medicinal Plant Extraction For LC-MS/MS Analysis

Pegaga samples were dried using Oven Model UF450. Dried specimen was grinded into powder by using mortar and pestle. 5mg of plant powder was extracted using modified folch extraction protocol. 4ml MeOH / Chloroform (1:1 v/v) mixture was added into powder plants. Then, mixtures were mixed thoroughly with 2 ml of 0.05M NaCl solution. Then, both Pegaga samples were centrifuged at 500g, 4 °C for 30 min. Both layers were transferred, vaporized and stored at -80°C. Before proceeding to LC-MS/MS analysis, plant extracts were re-dissolved in 1.5ml MeOH.

Liquid Chromatography-Tandem Mass Spectrometry

Briefly, 10 µl and 30 µl extract was introduced to Vanquish UHPLC system (Thermo Scientific, Waltham, MA, USA) coupled to ultra-high resolution Qq-Time-of-flight Impact II (Bruker, Billerica, MA, USA) at positive and negative electrospray ionization modes, respectively. Pentafluorophenyl column, Kinetex F5 (2.1 mm x 100 mm x 2.6 µm; Phenomenex, Torrance, California, USA) was utilized to perform chromatographic separation and maintained at 35 °C while mobile phase flow rate was maintained at 0.6 ml/min. During chromatographic separation, mobile phase A, mixture of deionized water with 0.1 % formic acid and 1 % ammonium acetate (NH_4AC) added while mobile phase B, consist of mixture of acetonitrile and methanol [6:4 v/v] with 0.1 % formic acid and 1 % NH_4AC added. The gradient elution was programmed to increase linearly from 1 % to 70 % of solvent B in 7 min, followed by 100 % solvent B from 7.1 to 10 min and maintained for 3 min. Later, the column was conditioned with initial gradient for 1 minute before the next sample injection. The data acquisition was set between *m/z* 50 and 1500. Positive and negative electrospray ionization voltage was set as 3.5 kV and -3.5 kV, respectively. Ion source gas temperature was set at 325 °C along with 10 L/min drying gas flow and nebulizer flow at 3 Bar. Mass spectrometer was calibrated with Tune Mix (Sigma-Aldrich, St Louis, MO, USA) before each batch analysis. Mass calibrant, sodium formate was introduced between 0.1-0.3 min during each acquisition. Post-acquisition acquired analytes *m/z* were calibrated against introduced sodium formate. For plant metabolome identification, similar chromatographic separation gradient was applied. Different collision energies were employed during molecule fragmentation was carried out as manufacturer's guidelines where molecules < *m/z* 200, 201-500, 501-750, and > 751 was predetermined as 10, 20 30 and 35eV, respectively.

Metabolite Identification

During the compound matching, a signal threshold was applied where compounds spectra above 1×10^3 intensity were selected for identification. The identification of extracted plant metabolome was based on metabolite fragmentation spectra matching using MS-Finder [22] referencing to UNPD (Nature product), PubChem (Biomolecule), KNApSAcK (nature product), NANPDB (Nature product) and PlantCyc (plant) database. Mass-to-charge ratio complement with the fragmented spectral and acceptable mass tolerance (at 5 ppm) allow the reveal of the plant metabolome identity. Filtering parameters and setting of MS-Finder for identifying putative metabolites are record in Table 2.

Table 2: Filtering parameter and setting for MS-FINDER.

Parameters & Setting	
Element Selection	Oxygen, Nitrogen, Phosphorus.
Local Databases	fooDB (Food), KNApSAcK (Natural Pro-duct), Pubchem (Biomolecules), PlantCyc (plant), DrugBank (Drug), ChEBI (Biomo-lecules), NANPDB (natural product), UNPD (natural product).
Molecular formula finder (Score: max 5)	4 score and above.
Structurer finder (Score: max 10)	7 score and above.

RESULTS

Morphological Identification

After comparative observation, both plant specimens were identified at the species level based on the morphological characteristics upon collection of the specimen. Each specimen was given a voucher number. Pegaga LYMOOI 046 was morphologically identified as *C. asiatica*, while Pegaga LYMOOI 069 was identified as *H. sibthorpioides*. The result of the morphological analysis on the two Pegaga species is presented in Table 3, the plant photographs and herbarium voucher are also presented in Figure 1.

DNA Barcoding Analysis

DNA extracted from the two Pegaga species was of high quality (ratios of absorbance, A260/280 and A260/230 ~1.80 and >1.90 respectively) and provided good yields of 123 ng/µl for *C. asiatica* and 100 ng/µl for *H. sibthorpioides*. Both samples with 3 different primers set exhibited 100 % PCR success. All the PCR products of the three DNA markers were successfully sequenced and good quality of bidirectional sequences were obtained.

The BLAST analysis performed with ITS was unable to identify the species with 100 % similarity. Even though it is not 100 % similar, the ITS region in the case of *C. asiatica* was still able to identify the species as the proposed plant species. For the other plant, ITS did not distinguish *H. sibthorpioides* from *C. asiatica*. In contrast, DNA barcoding analysis performed with the *rbc*L and *mat*K markers for both the samples successfully identified both the species, namely *C. asiatica* and *H. sibthorpioides*. BLAST results are recorded in Table 4.

Untargeted Metabolite Profiling

An UHPLC-MS/MS analysis-based approach was employed to profile the untargeted metabolite profiling of *C. asiatica* and *H. sibthorpioides*. MS-Finder, the window-based software that retrieve data from 15 databases was used to annotate the compound [23,24]. Through the parameter setting shown in Table 2, the results obtained for semi-quantitative screening of phytochemicals in the leaves of *C. asiatica* and whole plant of *H. sibthorpioides* are presented in Tables 5 and 6, respectively.

Preliminary phytochemical analysis revealed the presence of 24 putative compounds in *C. asiatica*: (RT) 1.62 min for nucleic acids, (RT) 1.12 min -1.97 min for N-containing compounds, (RT) 1.65 min - 3.72 min for phenolic compounds, (RT) 4.13 min - 11.51 min for terpenes compounds and (RT) 6.08 min - 11.60 min for other compounds. There are 30 putative compounds found in *H. sibthorpioides*: (RT) 0.99 min - 1.09 min for nucleic acids, (RT) 0.95 min - 11.17 min for N-containing compounds, (RT) 1.59 min - 8.21 min for phenolic compounds, (RT) 7.40 min for terpenes compound, (RT) 1.09 min - 5.59 min for other compounds. Results clearly indicate that fewer primary metabolites were detected in the plant extract. Thus, the secondary metabolites were mainly reported in this paper. The common metabolites compounds that detected in both the sample was shown in Figure 2. The sequence of major chemical classes found in *C. asiatica* are as follows: terpenes> phenolics> N-containing compounds> Other compounds. *H. sibthorpioides* contains more phenolics compounds than N-containing compounds and followed with other compounds and terpenes compounds.

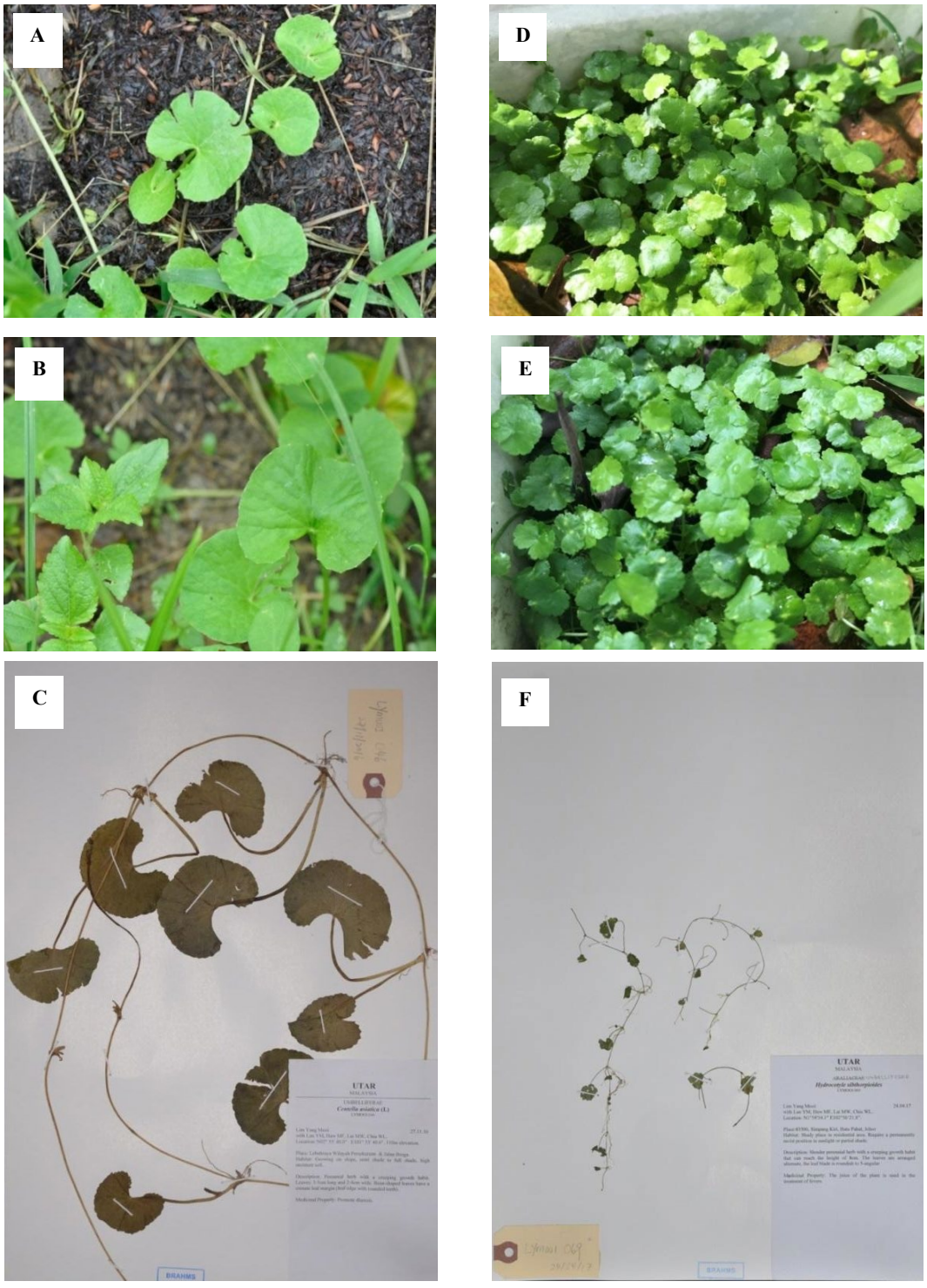

Figure 1: Photograph of *Centella asiatica* (L.) Urb, specimen LYMOOI 046 (A to C) (A) Habitat: Growing on the slope with high moisture soil and semi-shade or full shade. (B) Leaves with 3.0 – 8.0 cm diameter, reniform-shaped leaves and rounded teeth margin. (C) Herbarium voucher of LYMOOI 046. Photograph of *Hydrocotyle sibthorpioides* Lam., specimen LYMOOI 069 (D-F). (D) Habitat: Shady place in residential area. (E) Leaves with 0.5 - 1.0 cm diameter, clover-shaped leaves with shallow lobed margin. (F) Herbarium voucher of LYMOOI 069.

Table 3: Summary of morphological features of Pegaga species.

Features	***Centella asiatica***	***Hydrocotyle sibthorpioides***
Growth Habitat	Both Pegaga are found in slightly shade, moist to slightly wet habitat. They are perennial plants with creeping stem	
Leaf Shape	Reniform-shaped leaves	Clover-shaped leaves
Leaf Venation	Palmately veins	Palmately veins
Leaf Colour	Green	Light Green
Leaf Margin	Rounded teeth margin	Shallow lobed margin
Leaf Size	3.0 cm – 8.5 cm	0.5 cm – 1.0 cm
Petiole	Long Petiole, up to 20cm	Short petiole, up to 3cm

Table 4: Molecular identification of two Pegaga species. The species match in the reference database and the related identity value (Id. %) and accession obtained with the BLAST search are reported for the three barcode regions.

DNA Region	**Scientific Name/ Taxanomic Identification**					
	Centella asiatica (L.) Urb.			*Hydrocotyle sibthorpioides* Lam		
	Scientific Name	**Max. Id. (%)**	**Accession**	**Scientific Name**	**Max. Id. (%)**	**Accession**
*rbc*L	*Centella asiatica*	99	MN854377.1	*Hydrocotyle sibthorpioides*	100	KT589392.1
*mat*K	*Centella asiatica*	100	MN854377.1	*Hydrocotyle sibthorpioides*	100	KT589392.1
ITS	*Centella asiatica*	99	MH768338.1	*Hydrocotyle sibthorpioides*	96.1%	MH710672.1
				Centella asiatica	99.84%	JQ247225.1

Table 5: List of putative compounds in LYMOOOI 046, *Centella asiatica*, Precursor type: $(M+H)^+$, Plant Part: leaves.

Class	Precursor m/z(Da)	Retention Time (min)	Putative Compound Name	Ontology
Primary Metabolites				
Nucleic acids	268.1046	1.62	2-(6-aminopurin-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol	Adenosine
Secondary Metabolites				
N-containing compounds	120.0809	1.12	2,3-dihydro-1~{H}-indole	Indolines
	166.0867	1.13	3-amino-3-phenylpropanoic acid	Beta amino acids and derivatives
	188.0701	1.14	3-(1*H*-indol-3-yl)prop-2-enoic acid	Indoles
	205.0976	1.97	L-tryptophan	L-tryptophan
Phenolic	163.0392	1.65	3-Hydroxycoumarin	Hydroxycoumarins
	163.0389	2.06	8-hydroxy-2H-chromen-2-one	Hydroxycoumarins
	479.0821	2.74	Quercetin 3-O-β-D-glucuronide	Flavonoid-3-O-glucuronides
	163.0391	3.62	4-hydroxycoumarin	4-hydroxycoumarins
	287.0559	3.69	3,7,8-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one	Flavonols
	463.0873	3.72	Kaempferol 3-glucuronide	Flavonoid-3-O-glucuronides
Terpenes	487.3415	4.13	2α,19α-dihydroxy-3-oxo-12-ursen-28-oic acid	Triterpenoids
	471.3464	5.55	8-hydroxy-2,2,6a,6b,9,9,12a-heptamethyl-10-oxo-3,4,5,6,6a,7,8,8a,11,12,13,14b-dodecahydro-1H-picene-4a-carboxylic acid	Triterpenoids
	197.1319	6.08	1,4-dimethyl-7-prop-1-en-2-ylazulene	Guaianes
	353.2685	6.37	Tomentol	Sesquiterpenoids
	435.3257	8.06	4-[(1E,3Z,5E,7E,9E,11E,13E,15E)-17-hydroxy-3,7,12,16-tetramethylheptadeca-1,3,5,7,9,11,13,15-octaen-1-yl]-3,5,5-trimethylcyclohex-3-en-1-ol	Triterpenoids
	551.4251	8.99	3,5,5-trimethyl-4-[(1E,3E,5E,7E,9E,11E,13E,15E,17E)-3,7,12,16-tetramethyl-18-[(1S)-2,6,6-trimethylcyclohexa-2,4-dien-1-yl]octadeca-1,3,5,7,9,11,13,15,17-nonaenyl]cyclohex-3-en-1-ol	Xanthophylls
	569.4352	9.00	4-[(1E,3E,5E,7E,9E,11E,13E,15E)-16-(4,4,7a-trimethyl-2,4,5,6,7,7a-hexahydro-1-benzofuran-2-yl)-3,7,12-trimethylheptadeca-1,3,5,7,9,11,13,15-octaen-1-yl]-3,5,5-trimethylcyclohex-3-en-1-ol	Xanthophylls
	423.3619	10.48	Glochidone	Triterpenoids
	409.3832	11.51	Ferna-7,9(11)-diene	Triterpenoids
Others	187.1472	6.08	7-ethyl-1,4-dimethyl-4,5-dihydroazulene	Branched unsaturated hydrocarbons
	613.4824	8.93	(2S)-1-hydroxy-3-[(9Z,12Z)-octadeca-9,12-dienoyloxy]propan-2-yl (6Z,9Z,12Z,15Z)-octadeca-6,9,12,15-tetraenoate	Lineolic acids and derivatives
	429.3731	9.27	2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chromen-6-ol	Tocopherols
	760.5852	11.60	[2-(methylamino)ethoxy]({2-[(9Z)-octadec-9-enoyloxy]-3-(octadecanoyloxy)propoxy})phosphinic acid	Monomethylphosphatidylethanolamines

Table 6: List of putative compounds in LYMOOOI 069, *Hydrocotyle sibthorpioides*, Precursor type: $(M+H)^+$, Plant Part: whole plant.

Class	Precursor m/z(Da)	Retention Time (min)	Putative Compound Name	Ontology
Primary Metabolites				
Nucleic acids	136.0614	0.99	7*H*-purin-6-amine	Adenine
	268.1039	1.09	2-(6-aminopurin-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol	Adenosine
Secondary Metabolites				
N-containing compounds	160.1330	0.95	3-aminooctanoic acid	Beta amino acids and derivatives
	188.0700	1.96	3-(1*H*-indol-3-yl)prop-2-enoic acid	Indoles
	205.0969	1.99	L-tryptophan	L-tryptophan
	217.0969	2.42	3-(1-phenylethyl)imidazole-4-carboxylic acid	Carbonylimidazoles
	207.1246	8.63	2-amino-5-(diaminomethylideneamino)-2-(fluoromethyl)pentanoic acid	Alpha amino acids
	206.1410	11.17	N-(3-methylbut-2-enyl)-1,4,5,7-tetrahydropurin-6-imine	Imidazopyrimidines
Phenolic	163.0387	1.59	3-Hydroxycoumarin	Hydroxycoumarins
	163.0386	1.74	5-Hydroxycoumarin	Hydroxycoumarins
	163.0383	1.76	8-hydroxy-2H-chromen-2-one	Hydroxycoumarins
	355.1026	2.35	1-[3-(3,4-dihydroxyphenyl)prop-2-enoyloxy]-3,4,5-trihydroxycyclohexane-1-carboxylic acid	Quinic acids and derivatives
	449.1080	2.47	6-beta-D-Glucopyranosyl-3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one	Flavonoid C-glycosides
	433.1127	2.99	Kaempferol 3-O-alpha-rhamnoside	Flavonoid-3-O-glycosides
	303.0498	3.40	2-(3,4-dihydroxyphenyl)-3,7,8-trihydroxychromen-4-one	Flavonols
	595.1660	3.47	2-(3,4-dihydroxyphenyl)-5-hydroxy-3,7-bis[(3,4,5-trihydroxy-6-methyloxan-2-yl)oxy]chromen-4-one	Flavonoid-7-O-glycosides
	579.1710	3.55	5-hydroxy-2-(4-hydroxyphenyl)-7-[3,4,5-trihydroxy-6-[(3,4,5-trihydroxy-6-methyloxan-2-yl)oxymethyl]oxan-2-yl]oxychromen-4-one	Flavonoid-7-O-glycosides
	449.1070	3.56	Quercetin 3-O-L-rhamnoside	Flavonoid-3-O-glycosides
	465.1020	3.64	2-(3,4-dihydroxyphenyl)-6-beta-D-Glucopyranosyl-3,5,7-trihydroxy-4H-1-benzopyran-4-one	Flavonoid C-glycosides
	287.0543	4.12	3,5,7-trihydroxy-2-(3-hydroxyphenyl)-4H-chromen-4-one	Flavonols
	173.0595	5.79	2-hydroxynaphthalene-1-carbaldehyde	Naphthols and derivatives
	269.0810	5.84	5-hydroxy-3-(4-methoxyphenyl)chromen-4-one	4'-O-methylisoflavones
	337.1069	7.32	3-hydroxy-8-methoxy-3-methyl-2,4-dihydrobenzo[a]anthracene-1,7,12-trione	Angucyclines
	207.1019	8.21	(Z)-4-(4-hydroxy-3-methoxyphenyl)-3-methylbut-3-en-2-one	Hydroxycinnamic acids and derivatives
Terpenes	275.2014	7.40	(3E,5E,7E)-8-[(4R)-4-hydroxy-2,6,6-trimethylcyclohex-1-en-1-yl]-6-methylocta-3,5,7-trien-2-one	Sesquiterpenoids
Others	229.1550	1.09	Fatty acid methyl esters	Fatty acid methyl esters
	177.0539	3.30	3-(1,3-benzodioxol-5-yl)prop-2-enal	Benzodioxoles
	181.1220	4.52	4-hydroxy-3-methyl-2-[(2E)-pent-2-en-1-yl]cyclopent-2-en-1-one	Secondary alcohols
	293.2115	5.47	10-methoxyheptadec-1-en-4,6-diyne-3,9-diol	Long-chain fatty alcohols
	295.2270	5.59	(6Z,9Z)-11-(3-pentyloxiran-2-yl)undeca-6,9-dienoic acid	Long-chain fatty acids

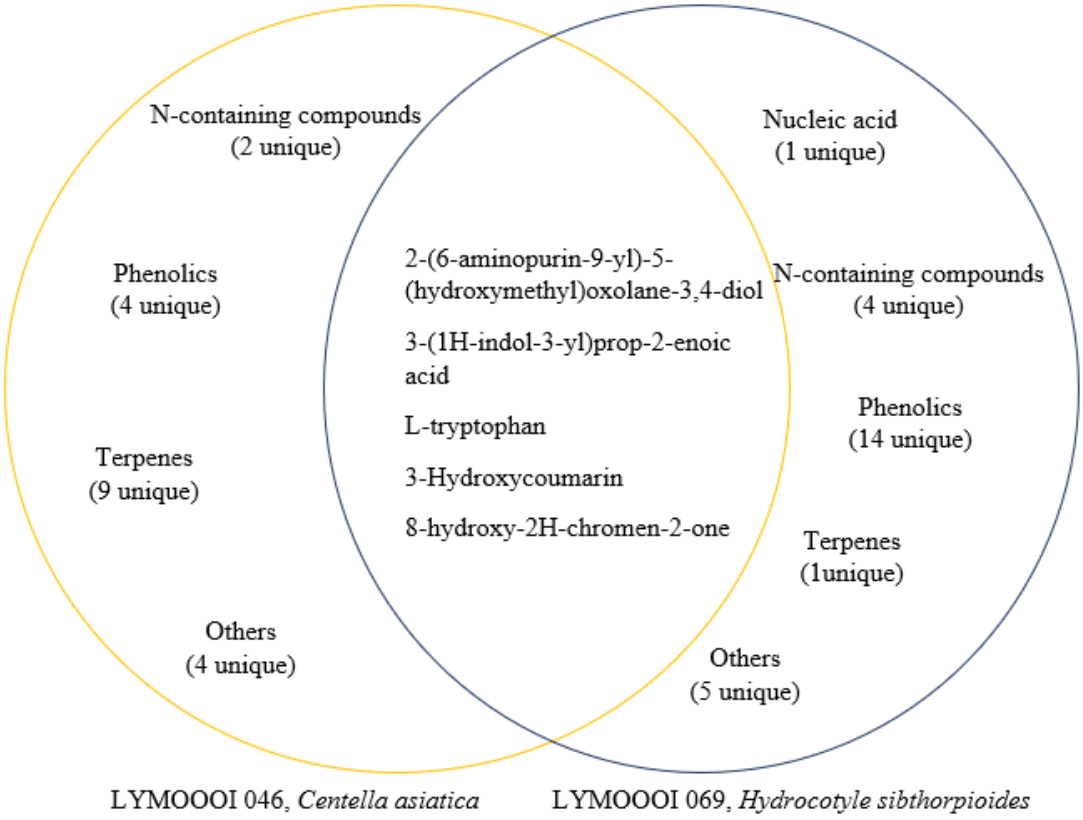

Figure 2: Venn diagram showing the common metabolites that shared between the *Centella asiatica* and *Hydrocotyle sibthorpioides.*

DISCUSSION

Morphological Identification

According to Ren et al. [25], the morphology identification method is suitable for the specimens with unbroken leaves due to its fast, direct and convenient characteristics. The morphological characteristics of the two Pegaga species were distinct upon close investigation. Based on the in situ visual morphological traits, the two Pegaga species collected were identified as *C. asiatica* and *H. sibthorpioides*. Plant phenotype showed a clear difference between *C. asiatica* and *H. sibthorpioides* using the traits of leaf shapes, leaf size and petiole. Leaves of *C. asiatica*, reniform in shaped, diameter range from 3 cm to 8.5 cm, which is much bigger in size as compared to *H. sibthorpioides* that only have leaves diameter of 0.5 cm to 1cm, in clover like shaped. The former can grow up to 20 cm but the latter are low-growing groundcover plants which grow up to 3 cm height from land. Similar morphological characteristics was observed by previous researcher, reporting that the leaf margin of leaves for *C. asiatica* has more dentate [26], leaves' diameter is bigger, long petiole while *H. sibthorpioides* has shallow lobed leaves margin with smaller leaves' diameter and short petiole [27]. The phenotypes of both Pegaga clearly differentiate one from another. Both Pegaga species were preserved and mounted as herbarium. Approximately 390 million of specimen of preserved plants, algae, fungi and other related taxa are currently reside in 3000 herbaria over 176 countries [28] and it serves the roles for taxonomy and systematics, anthropology, education to ecology climate change research [29]. However, morphological identification has some major drawbacks, such as the key morphological appearance are only observable during flower or fruit stage but not the vegetative state; phenotypic plasticity and insufficient taxonomy expertise [30]. Hence, medicinal plant identification seems to be challenging by using only morphological characteristics.

DNA Barcoding

DNA barcoding has been proposed as a rapid tool for identifying species and becomes one of the ways to overcome the problems of plant identification. CO1 has been developed as a universal DNA barcode for animals and algae, however in plants, no a gold standard barcode marker has been developed [31]. The successful discrimination of plant species by the gene region may vary in plants [32]. So far, despite all the initiative, no one locus or combination of locus has proven to be universal DNA barcode in plant

species identification [33]. Different barcode are reported to success in identify different taxa, *rbc*L locus for mangrove [34] and palmae [35]; *mat*K locus for Lamiaceae [36], Orchidaceae [37] and *Vachellia* species [38]; ITS for Alnus [39] and Ficus [40].

High quality DNA was obtained from both the Pegaga samples and the success of DNA barcoding is dependent on the primer universality [41]. Three primer pairs (*rbc*L, *mat*K and ITS) that were tested resulted in 100 % amplification and sequencing success, which suggests the importance of selection of primer in barcoding studies. According to the results obtained, ITS was less suitable to discriminate both Pegaga species as compared to *rbc*L and *mat*K. Identification using the core barcode of *rbc*L and *mat*K for both Pegaga matched with morphological identification to the species level, which is consistent with previous study [42]. In contrast, this finding is inconsistent with previous study done by Tripathi et al. [43] and Xu et al. [44], who showed that ITS shows better discriminatory power than *rbc*L and *mat*K. This study as well as other barcoding studies found that the *rbc*L has high universality in terms of good quality bidirectional sequencing. Many researches have indicated that *mat*K is a key marker discriminating specific plant species [45], although some of other laboratories have reported low amplification and sequencing success rate of *mat*K region [46,47]. The findings presented in this study did not observe any of the difficulties associated with *mat*K as a barcode, therefore this region can be considered as an effective barcode for Pegaga species. ITS have been shown to be an effective single barcode region when it comes to *Paphiopedilum* species [48] and Orchidaceae [44]. In spite of the fact that ITS was proposed as a complementary universal DNA barcode [49], its usage in DNA barcoding was still questioned in some groups [50,51]. There are disadvantages that drawback its widespread use. ITS is prone to contamination from fungal epiphytes or endophytes [52], presence of paralogous copies that may occur naturally in the cell [53], and exhibits incomplete concerted evolution [54]. ITS generally offers more resolution at low taxonomic level [55], however, ITS showed less favourable as it is unable to discriminate the sample of *H. sibthorpioides* to species level in this study. Thus, in contrast to *rbc*L and *mat*K, ITS is not suitable for DNA barcoding in Pegaga species, although it may be useful in other plants.

Untargeted Metabolite Profiling

Plant secondary metabolites served to defend the plants against stress, insect herbivores and also as mediators of interactions with mutualists and competitors [56]. Untargeted metabolite profiling is a 'discovery mode' process, aiming to analyse all known and unknown detectable metabolites [57]. *C. asiatica* and *H. sibthorpioides* appear to be chemically very different. As illustrated in Tables 5 and 6, the former is high in both terpenes and phenolic compounds while large numbers of phenolic compounds present in the latter.

This study showed that *C. asiatica* is high in both terpene and phenolic compounds. Studies of Maulidiani et al. [17] revealed that compound kaempferol and quercetin were the major phenolics found in the Pegaga extracts. Quercetin 3-O-β-D-glucuronide and Kaempferol 3-glucuronide were identified in *C. asiatica* extract. Study from Shankaran *et al.* [58] determined the property of Kaempferol 3-glucuronide towards antioxidants. Quercetin 3-O-β-D-glucuronide has been reported for anti-aging [59], anti-cancer [60] and anti-inflammatory properties [61]. Five out of seven terpene compounds detected were triterpenoids. According to Zheng and Qin [62], triterpenoids is the major components of *C. asiatica*, it is known for high contents of pentacyclic triterpenoids (C30), especially asiaticoside, madecassoside, asiatic acid and madecassic acid are abundant in *C. asiatica* [63]. In the present research, five pentacyclic triterpenoids as shown in Table 5 were obtained, however, the four major pentacyclic triterpenoids (C30) mentioned (asiaticoside, madecassoside, asiatic acid and madecassic acid) above were absent. Different metabolite content can be explained by previous studies which indicate that exposure of full day light increases the contents of triterpenoids of *Centella* as compared to those grown in shade [64,65]. Research by Puttarak and Panichayupakaranant [66] showed that the condition of cultivation, and harvesting period affect the active compounds of *C. asiatica*. This is further supported by other researchers which showed that secondary metabolites contents may vary depending on growth location and subjected to the environmental factor [67,68]. Ursane type pentacyclic triterpenes, namely 2α, 19α-dihydroxy-3-oxo-12-ursen-28-oic acid identified at m/z 487.3415 with fragment ion of 187.1473 and 405.3138 was first time reported in *C. asiatica* for this study. The compound showed potent inhibitory activity against HIV-1 protease [69] and was found to exert anti-diabetic effect [70].

In contrast to *C. asiatica*, only one terpenes compound with unknown function was detected in *H. sibthorpioides* extract. Instead, the presence of phenolic compounds in *H. sibthorpioides* was high in this study, as 16 various phenolic compounds as shown in Table 6 were reported, in which flavonoid glycosides is the major compound. The result is in agreement with previous work indicating flavonoid glycosides is the chemical marker for *H. sibthorpioides* [17]. Result from Kumari et al. [71] demonstrated the promising antioxidant properties of *H. sibthorpioides* extract. Both compounds of kaempferol 3-O-alpha-rhamnoside and quercetin 3-O-L-rhamnoside were found to be present in these Pegaga species. In other studies, kaempferol 3-O-alpha-rhamnoside extracted from *Bauhinia megalandra* leaves was reported to show inhibition of glucose intestinal absorption and suggested to be the competitive inhibitor of intestinal SGLT1 cotransporter [72]. Whereas, quercetin 3-O-L-rhamnoside isolated from *Rosa chinensis* was reported by Qing et al. [73] to possess antioxidant properties.

CONCLUSIONS

Taken together, the combination of morphological characteristics, DNA barcoding and metabolite profiling can discriminate both Pegaga species. The core gene regions of *rbc*L and *mat*K successfully identified the Pegaga species but not the ITS gene region. Untargeted metabolite profiling revealed the presence of 24 putative compounds in *C. asiatica* and 30 putative compounds in *H. sibthorpioides*. The results showed that both the Pegaga species is chemically different as the former is high in both terpenoid and phenolic compounds, and the latter is high in phenolic compounds especially flavonoid glycosides.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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