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MORPHOLOGICAL CHARACTERISTICS, DNA BARCODING AND METABOLITE PROFILING OF *Oldenlandia diffusa* (Willd.) Roxb. AND *Oldenlandia corymbosa* L.

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Abstract

Oldenlandia diffusa (Willd.) Roxb. is a well-known traditional Chinese medicinal herb and frequently replaced by *Oldenlandia corymbosa* L. However, their roles and efficacies are not the same. Therefore, the purpose of this study was to distinguish these two plants based on morphology and DNA barcodes, and to reveal their putative compounds. Wild *O. diffusa* and *O. corymbosa* were collected from Johor and Negeri Sembilan in Malaysia, respectively. The morphological characteristics of both plants were recorded during plant collection. DNA barcodes of ribulose-1,5-bisphosphate carboxylase (*rbc*L), maturase K (*mat*K), and nuclear ribosomal internal transcribed spacer (ITS) were used to identify these plants up to species level. Non-targeted metabolite profiling was performed to determine the putative compound of both plants. Results obtained for *rbc*L, *mat*K, and ITS DNA barcoding analysis showed 100 % similarity of *Oldenlandia corymbosa* L. Results obtained for *mat*K DNA barcoding analysis showed 99.64 % similarity of *Oldenlandia diffusa* (Willd.) Roxb. LC-MS/MS analysis revealed a total of 42 and 12 primary and secondary putative compounds detected in *O. corymbosa* and *O. diffusa* extracts, respectively. Overall, we currently recommend the use of both morphological and DNA barcoding for plant identification. In the future, we proposed to design a primer to increase primer specificity and further confirm the structure and biological function of potential putative compounds.

INTRODUCTION

Nowadays, there are about 70 % to 80 % of the world population relies mainly on herbs for their primary medicines [1]. *Oldenlandia diffusa* (Willd.) Roxb. (Family: Rubiaceae) is a famous folk medicine in Southern China, used to treat hepatitis, tonsillitis, sore throat, appendicitis, urinary tract infection and liver, lung, and stomach malignant tumours [2]. In the market, *O. diffusa* is frequently adulterated by a related species *Oldenlandia corymbosa* L. (Synonym: *Hedyotis corymbosa* (L.) Lam) [3, 4].

O. diffusa is reported as the traditional Chinese herb by Chinese Pharmacopoeia [5]. Based on the theory of Traditional Chinese Medicine (TCM), *O. diffusa* is claimed to possess functions of heat-clearing, detoxification, increase blood circulation, and remove blood stasis [6]. Besides

distributed in China, it is also an annual herb that widely distributed in the northeast Asian countries [7]. It is commonly used in the Orient and tropical Asia to make teas and botanicals for the treatment of heat, elimination of toxins and stimulation of diuresis to remove wetness-evil [3, 4]. Multiple biological activities are exhibited by *O. diffusa*, such as antitumour, chemopreventive, anti-angiogenic, anti-inflammatory, antioxidant, and proapoptotic effects [7, 8].

Although *O. diffusa* and *O. corymbosa* have similar morphology characteristics, their roles and efficacies are not the same [9]. Based on the TCM theory, functions of *O. corymbosa* are to clear heat and toxins, improve blood circulation, promote diuresis, and relieve strangury (urinary obstruction). Several reports also mentioned its therapeutic effects on digestive tract lymphosarcoma and carcinoma of the liver and larynx, as well as used to treat appendicitis, hepatitis, pneumonia, cholecystitis, urinary infection, cellulites, and snake bite [10]. In Malaysia, *O. diffusa* is named as *Rumput jinga*, whereas *O. corymbosa* is named as *Siku-siku*, *Rumput mutiara*, *Bunga telur belangkas*, *Daun mutiara*, and *Lidah ular*. Both *Oldenlandia* species belong to Rubiaceae family and these small straggling herbs can be found among the weed species throughout Malaysia [11].

Many investigations have been developed to differentiate *O. diffusa* from *O. corymbosa* such as fluorescence microscopy [12], morphology analysis [13, 14], DNA sequencing [15], thin layer chromatography [41], high performance liquid chromatography [3], etc. According to Yik et al. [16] morphological characteristics of *O. diffusa* and *O. corymbosa* are slightly different, hence, they could have distinct chemical compounds. However, those previously studied lacked the metabolite profile of *O. diffusa* and *O. corymbosa*, which provided the basis for the establishment of *O. diffusa* and *O. corymbosa* quality standards in the future. Furthermore, herbarium vouchers should be specifically collected and accessioned to support a research project (e.g., genetic analysis of a taxon) and researchers may later obtain the specimen for examination and further study [17].

Correct identification of herbal plant is the foundation for safety use of herbal medicines and products. Without proper identification as a starting point, the safety for using medicinal herbs cannot be guaranteed [18, 19]. In order to ensure the correct use of both plants, it is necessary to develop an efficient method and reliable DNA markers to distinguish them. Morphological and molecular approaches are the ideally methods used for plant identification [20]. The morphological identification is traditionally performed by taxonomists [21]. Although these two plants displayed some morphological differences, it is still hard for people without taxonomy expertise to distinguish them based on morphology. Recently, non-taxonomist experts have proposed DNA barcoding for identification because it can be easily repeated [22]. DNA barcoding offers an accurate alternative to morphological identification, and it is frequently used when identification using macroscopic methods became challenging. It is a species identification tool that uses internationally agreed protocols, and DNA fragments to create a global database of living organisms [23]. Identification at the DNA level provides more reliability because DNA is a stable macromolecule that is not affected by external factors and is found in all tissues [24]. Besides, it also can be utilized to identify and discriminate species in any developmental or processed stage from which DNA can be extracted [25]. Meanwhile, each DNA barcoding of the plant should be accompanied with a herbarium voucher; this will provide high quality databases [26].

The correct use of plants is also important because of the existence of unique types of compounds in different plant species [27]. The knowledge about chemical composition of medicinal herb is important for constructing the identity, purity, efficacy, and safety of herbal medicinal product [28]. Metabolite profiling is one of the most powerful methods and is mainly used for non-targeted metabolite analysis [29]. Non-targeted analysis can collect data without pre-existing knowledge [30] and can provide a more comprehensive and unbiased approach for metabolite analysis [31]. Hence, the objectives of this study were to find the ideal ways for distinguishing the identity of wild *Oldenlandia* species and determine the putative compounds.

MATERIALS AND METHODS

Plant Material

A plant samples labelled as LYMOOI 066 and LYMOOI 073 were collected from Broga Lenggeng, Negeri Sembilan (N 02° 55' 55.5" E 101° 55' 28.1") and Batu Pahat, Johor (N 01° 58' 54.1" E 102° 56' 21.8"), respectively. Both are wild plants. They were collected under sunlight and moist soil conditions. Macroscopic identification was done based on external features of plants (leaves, stem, flower, and whole plant), and followed by harvesting the plant. Each plant sample was collected individually and placed in different sterile plastic bags for DNA isolation and LC-MS/MS extraction. All plant samples were delivered immediately to laboratory after collection and refrigerated at -20 °C prior to analysis.

Morphological Identification

Both *Oldenlandia* species were identified by herbalist. The morphology of inflorescences, plant height, leaves sizes, and leaves arrangement were used to identify the plants. The plant's height and leaf size were measured with a measuring tape. The characteristics of *Oldenlandia* species were recorded in accordance with the template provided by Forest Research Institute Malaysia (FRIM).

Preparation of Herbarium Voucher

The herbarium vouchers were prepared according to the methods provided by Forest Research Institute Malaysia (FRIM). *Oldenlandia* species was freshly collected as whole or part of the plant along with flowers. It was carefully cleaned and directly placed in contact with newspapers and pressed with a wooden board; these plants were further dried in the oven. Apart from this, each specimen was assigned with an official herbarium labelling code. Once the dried specimen was ready, they were sent to Perdana Botanical Garden Kuala Lumpur for mounting, verification, and storage. The taxonomy data collection was recorded in the database BRAHMS (Botanical Research and Herbarium Management System). The herbarium voucher numbers are LYMOOI 066 and LYMOOI 073, respectively.

DNA Barcoding

DNA Extraction

Oldenlandia species was sampled for DNA barcoding. Young leaves were chosen for the DNA extraction. Prior to DNA extraction, all the leaves were first wiped with 75 % ethanol to remove adhering dirt and sand. The leaves collected from individual plant samples were ground in liquid nitrogen and the total DNA was extracted using the GeneJET Plant Genomic DNA Purification Kit (Thermo Scientific) based on the standard protocol. The extracted DNA was kept at -20 °C for polymerase chain reaction (PCR).

Polymerase Chain Reaction (PCR) Amplification

Samples were amplified by PCR using the primer pairs (listed in Table 1 for *rbc*L, *mat*K and ITS) provided by Phire Plant Direct PCR Mastermix (Thermo Scientific). PCR was performed using Veriti® 96 Well Thermal Cycle (Applied Biosystems). Successfully amplified DNA fragments were then visualized using 1.5 % agarose gel electrophoresis. 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 sending for DNA sequencing.

DNA Sequence Analysis

Primers used for PCR amplification were also used in DNA sequencing reactions (Table 1). Bidirectional sequencing data was aligned using MEGA 7.0. Bioinformatics tool was then used to identify the identity of the species. Aligned and consensus sequences for each locus of each plant sample were searched in GenBank database through BLAST procedure. Top matching hit of maximum identity (>95 %) of each sample was taken as the barcoding identification. If the result indicated that the sample is different with the prior assigned taxon, it was flagged as a possible error and the sample was then compared with descriptions and herbariums specimens of the species involved, by using morphological characteristics to confirm whether an error had been made.

Table 1. Primer sequences and thermocycling condition

Region	Primer	Sequence (5'-3')	Thermocycling condition	Reference
*rbc*L	rbcLa-F	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.	[83]
	rbcLajf634R	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.	[83]
	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.	[84]
	ITS_8P	CAC GCT TCT CCA GAC TAC A		

Non-targeted Metabolite Profiling

Sample Preparation and Extraction for LCMS-MS Analysis

Whole plant sample of LYMOOI 066 and leaves sample of LYMOOI 073 were dried in oven (Model UF450) with temperature of 40 ºC for 5 days and grinded into powder by using mortar and pestle. Ground sample of 5 ±0.5 mg were exhaustively extracted based on Folch extraction protocol [32] with appropriate modifications [33–35]. Briefly, powdered plants were extracted using solvent at a final ratio of 100% methanol: 100% chloroform: 0.05 M NaCl solution equal to 1:1:1 v/v/v. Centrifugation of a sample at 500g and applied at 4 ºC for 30 min. Both upper (hydrophilic compounds) and lower (hydrophobic compounds) layers were transferred, vaporized, and stored at -80 °C until analysis. Prior to the LC-MS/MS analysis, plant extracts were re-dissolved in 1.5 mL methanol.

Liquid Chromatography-Tandem Mass Spectrometry

The LC-MS/MS system consists of Vanquish UHPLC system (Thermo Scientific, Waltham, MA, USA) coupled to ultra-high resolution Qq-Time-of-flight Impact II (Bruker, Billerica, MA, USA) was used for the metabolite profiling. In short, 10 µL and 30 µL extract was inserted to the system at positive and negative electrospray ionization modes, respectively. The chromatographic separation was done on a pentafluorophenyl column, Kinetex F5 (2.1 mm x 100 mm x 2.6 µm; Phenomenex, Torrance, California, USA). The column temperature was kept at 35 °C. The flow rate was set at 0.6 ml/min. For separation, mobile phases A (mixture of deionized water with 0.1 % formic acid and 1 % ammonium acetate (NH_4AC)) and mobile phase B (mixture of acetonitrile and methanol [6:4 v/v] with 0.1 % formic acid and 1 % NH_4AC) were used. The gradient elution was set 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 carried on for 3 min. Then, the column was equilibrated with initial gradient for 1 minutes before the next sample injection. 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 calibration was enabled using Tune Mix (Sigma-Aldrich, St Louis, MO, USA) before each batch analysis. Mass calibrant, sodium formate was presented between 0.1-0.3 min during each acquisition. Post-acquisition, obtained analytes m/z were calibrated against sodium formate. Various collision energies used during molecule fragmentation was carried out based on manufacturer's guidelines where molecules < m/z 200, 201-500, 501-750, and > 751 was pre-established at 10, 20, 30 and 35 eV, respectively.

Metabolite Identification

Signal threshold was set above 1×10^3 intensity during compound matching. MS Finder [36] matched to database such as UNPD (Nature product), PubChem (Biomolecule), KNApSAcK (nature product), NANPDB (Nature product) and PlantCyc (plant) was used to identify the plant compound (Table 2). Mass-to-charge ratio compliment with the fragmented spectral and acceptable mass tolerance (at 5 ppm) was adopted to determine the plant compounds.

Table 2. MS-Finder Parameters for selected potential LC-MS/MS putative compounds

Parameters & setting	
Element Selection	Oxygen, Nitrogen, Phosphorus.
Local Databases	fooDB (Food), KNApSAcK (Natural Product), PubChem (Biomolecules), PlantCyc (plant), DrugBank (Drug), ChEBI (Biomolecules), 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 AND DISCUSSION

Morphological Identification

Due to their similar morphology, *O. diffusa* and *O. corymbosa* are often misidentified. During plant collection, sample LYMOOI 073 identified as *Oldenlandia diffusa* (Willd.) Roxb. and sample LYMOOI 066 identified as *Oldenlandia corymbosa* L. were based on morphological differences in terms of the number of the inflorescence. The number of inflorescences is the main differentiating phenotype in these plants. The result of the morphological analysis on *Oldenlandia* species is presented in Table 3, the plant photographs and herbarium voucher are presented in Figure 1.

Table 3. Morphological analysis for *Oldenlandia* species

	Oldenlandia corymbosa	***Oldenlandia diffusa***
Growth habitat	Both *Oldenlandia* are annual slender herbs. Growing habitat is at semi-shade to full sunlight area.	
Leaves arrangement	Arrange oppositely	
Inflorescences	Inflorescences 2 to 4 (usually 3) in axillary with pedicle 0.4-1.2 cm long, peduncle 0.8 cm to 1.4 cm long	Inflorescence terminal with short peduncle of 0.4 cm to 1.0 cm
Plant height	5 cm to 20 cm tall	15 cm to 50 cm tall
Leaves sizes	2-5 cm long, 1-1.5 cm width.	1-3 cm long, 0.1-0.4 cm width

Figure 1. Photograph of *Oldenlandia corymbosa* L., specimen LYMOOI 066 (A-C) (A) Habitat: Growing at semi-shade to full sunlight area. (B) Inflorescences 2 to 4 in axillary with pedicle 0.4 -1.2 cm long, peduncle 0.8 cm to 1.4 cm long. (C) Herbarium voucher of LYMOOI 066. Photograph of *Oldenlandia diffusa* (Willd.) Roxb, specimen LYMOOI 073 (D-F). (D) Habitat: Growing at semi-shade to full sunlight area. (E) Inflorescence terminal with short peduncle of 0.4 cm to 1.0 cm (F) Herbarium voucher of LYMOOI 073

DNA barcoding Analysis

High quality product (ratios of absorbance, A260/280 and A260/230 ~1.80 and >1.90 respectively) of PCR was yielded from the amplification of *rbc*L, *mat*K, and ITS. The generated query sequences were matched with the references sequences in BLAST. The query sequences of *rbc*L, *mat*K, and ITS successfully identified sample LYMOOI 066 as *O. corymbosa* up to species level for 100 % similarity. For sample LYMOOI 073, barcode region of *mat*K showed only 99.64 % similarity as *O. diffusa.* Barcode regions of *rbc*L and ITS failed to identify sample LYMOOI 073 as *O. diffusa*. This indicated that sample of LYMOOI 073 can only be identified up to genus level by using barcode regions of *rbc*L, *mat*K, and ITS (Table 4).

Non-targeted Metabolite Profiling

Non-targeted LC-MS/MS data analysis was used to reveal the putative compounds detected in both plants. In this study, negative mode was excluded due to no result. MS-FINDER software that embedded with 15 databases was used to identify the putative compounds [37]. The putative compounds of both plants were grouped into two categories, namely primary and secondary metabolites [38]. Based on the parameter settings shown in Table 2, 12, and 42 putative compounds were detected from *O. diffusa* leaves extract and *O. corymbosa* whole plant extract, respectively (Tables 5 and 6). One nucleic acid, 10 phenolic compounds and 1 other compound were obtained for *O. diffusa* (Table 5). One nucleic acid, 3 proteins, 2 pigments, 3 nitrogen- containing compounds, 16 phenolic compounds, 7 terpenes, 10 other compounds were found in *O. corymbosa* (Table 6). There were 8 same putative compounds detected in both *Oldenlandia* species (Figure 2), the same compounds identified were 2-(6-aminopurin-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol, Desmethyldiaportinol, 4-hydroxynaphthalene-1,2-dione, (E)-5-(4-hydroxyphenyl)-3-oxopent-4-enoate, 3-Hydroxycoumarin, 2,3-dihydrofuro[3,2-g]chromen-7-one,4-(2-methoxyphenyl)-2-oxobut-3-enoic acid and 2-(1-hydroxycyclopentyl)- 2-phenylacetic acid.

DISCUSSION

Morphological Identification

The taxonomy differentiation of *O. diffusa* and *O. corymbosa* is extremely challenging [39] because they look alike in fresh [40]. According to Sun et al. and Lau et al. [5, 41], *O. diffusa* and *O. corymbosa* can be easily misidentified. Based on the external observations, the two plants are similar in terms of plant size, leaves arrangement, colour of flowers, and growth habitat. People often confuse these two plants with those common morphological characteristics. To differentiate them, *Flora Republicae Poluparis Sinicae* indicated three important external morphologies of *O. diffusa*. It has solitary flowers on thick and short pedicels (2 to 4 cm in length) growing from cylindrical stems. In contrast, *O. corymbosa* differs in having corymbosa inflorescences on slender peduncles (8 to 15 mm in length) growing from tetragonal stems [42]. In this study, sample LYMOOI 073 and sample LYMOOI 066 can be identified as *O. diffusa* and *O. corymbosa*, respectively, by observing the inflorescence, plant height, and leaves size (Table 3). In terms of configuration of the flowers of *O. diffusa* and *O. corymbosa*, our macroscopic observations are similar to the reports of previous researchers [43]. *O. diffusa* has inflorescence terminal with short peduncle of 0.4 cm to 1.0 cm, while *O. corymbosa* has 2 to 4 (usually 3) of inflorescences in axillary with peduncle of 0.8 cm to 1.4 cm. However, morphological criteria are often subtle and ambiguous [44], it is essential to have herbarium specimens for verification or a solid database that guarantees the reference specimen is accurately identified by a taxonomic expert [45]. To evaluate the accuracy of herbarium image, Carranza-Rojas et al. [46] suggested that it is vital to study the combination of herbarium sheets with photos of plants in the field. In present study, both *Oldenlandia* species were successfully identified by visual examination, plant photography and herbarium voucher (Table 3 and Figure 1).

DNA Barcoding Analysis

DNA barcoding is an important tool for species identification [47]. An ideal DNA barcode should be routinely retrievable with a single primer pair, be resigned to bidirectional sequencing with little requirement for manual editing of sequence traces and offer maximal discrimination among species [48]. Consortium for the Barcode of life (CBOL) has claimed that the *rbc*L and *mat*K are the main barcode markers for accurate identification of plants and trees [49]. The *rbc*L and *mat*K were selected to focus because *rbc*L regarded as most characterized plastid region in GenBank, has widespread representation from all major groups, and will thus give a solid baseline for comparison with other plastid regions [50] and *mat*K region has high rate of nucleotide substitutions [51] or restructuring of the locus [52]. In addition, nuclear ribosomal internal transcribed spacer (ITS) is used as the complementary marker [53]. Guo et al. [54] indicated that ITS has benefit of high PCR amplification success and it gives good intra- and interspecific variation distribution patterns. In this study, *rbc*L, *mat*K, and ITS were applied for *Oldenlandia* species identification. The results suggested that the success rate for accurately identify *O. corymbosa* was higher than *O. diffusa*. The BLAST results of *rbc*L, *mat*K, and ITS showed that sample LYMOOI 066 was successfully identified as *Oldenlandia corymbosa* L. It indicates that LYMOOI 066 can be identified up to species level. However, BLAST

Table 4. BLAST search results of two *Oldenlandia* species. For the *rbc*L, *mat*K, and ITS regions, query sequences that match related species and their identity value (Id. %) and accession are reported

Scientific name/ Taxanomic identification	DNA region								
	*rbc*L			*mat*K			ITS		
	Scientific name	Max. Id. (%)	Accession	Scientific name	Max. Id. (%)	Accession	Scientific name	Max. Id. (%)	Accession
Oldenlandia corymbosa	*Oldenlandia corymbosa*	100	NC_057983.1	*Oldenlandia corymbosa*	100	NC_057983.1	*Oldenlandia corymbosa*	100	AM939500.1
Oldenlandia diffusa	*Oldenlandia corymbosa*	100	NC_057983.1	*Oldenlandia diffusa*	99.64	NC_057985.1	*Oldenlandia corymbosa*	100	AM939500.1

Table 5. List of putative compounds in LYMOOI 073, *O. diffusa*, Precursor type: $(M+H)^+$, Plant Part: leaves

Taxa	Kind of Metabolites	Class	Precursor m/z(Da)	Retention Time (min)	Putative Compound Name	Ontology
LYMOOI 073	Primary	Nucleic acids	268.1043	1.64	2-(6-aminopurin-9-yl)-5-(hydroxymethyl)oxolane-3,4- diol	Adenosine
	Secondary	Phenolics	193.0495	1.34	7,8-dihydroxy-6-methyl-2H-chromen-2-one	7,8-dihydroxycoumarins
			235.0605	2.22	4-methyl-2-oxo-2H-chromen-7-yl 2-hydroxyacetate	Coumarins and derivatives
			253.0708	2.37	Desmethyldiaportinol	Isocoumarins and derivatives
			175.0389	2.37	4-hydroxynaphthalene-1,2- dione	Naphthoquinones
			207.0652	2.62	(E)-5-(4-hydroxyphenyl)-3-oxopent-4-enoate	Hydroxycinnamic acids and derivatives
			163.0387	2.79	3-Hydroxycoumarin	Hydroxycoumarins
			249.0757	2.94	4,9-dimethoxy-2,3-dihydrofuro[3,2-g]chromen- 7-one	Psoralens
			189.0543	2.95	2,3-dihydrofuro[3,2- g]chromen-7-one	Psoralens
			287.0546	3.68	3,5,7-trihydroxy-2-(3-hydroxyphenyl)-4H-chromen- 4-one	Flavonols
			207.0642	3.92	4-(2-Methoxyphenyl)-2-Oxobut-3-Enoic Acid	Cinnamic acids and derivatives
		Others	221.1172	6.16	2-(1-hydroxycyclopentyl)-2-phenylacetic acid	Benzene and substituted derivatives

Table 6. List of putative compounds in LYMOOI 066, *O. corymbosa*, Precursor type: $(M+H)^+$, Plant Part: whole plant

Taxa	Kind of Metabolites	Class	Precursor m/z(Da)	Retention Time (min)	Putative Compound Name	Ontology
LYMOOI 066	Primary	Nucleic acids	268.1035	1.01	2-(6-aminopurin-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol	Adenosine
		Proteins	116.0705	0.63	(2Z)-2-(methylamino)but-2-enoic acid	Alpha amino acids
			166.0860	1.11	3-amino-3-phenylpropanoic acid	Beta amino acids and derivatives
			207.1245	10.34	2-amino-5-(diaminomethylideneamino)-2-(fluoromethyl)pentanoic acid	Alpha amino acids
		Pigments	551.4257	8.98	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
			607.2923	9.36	Methyl pheophorbide a	Chlorins
	Secondary	N containing compounds	188.0700	1.11	3-(1*H*-indol-3-yl)prop-2-enoic acid	Indoles
			120.0805	1.12	2,3-dihydro-1~{H}-indole	Indolines
			205.0973	1.97	L-tryptophan	L-tryptophan
		Phenolics	147.0439	0.60	3-phenylprop-2-ynoic acid	Benzene and substituted derivatives
			175.0387	1.95	2-Hydroxy-1,4- naphthoquinone	Naphthoquinones
			253.0707	2.36	Desmethyldiaportinol	Isocoumarins and derivatives
			175.0387	2.62	4-hydroxynaphthalene-1,2-dione	Naphthoquinones
			207.0652	2.63	(E)-5-(4-hydroxyphenyl)-3-oxopent- 4-enoate	Hydroxycinnamic acids and derivatives
			163.0385	2.75	8-hydroxy-2H-chromen-2-one	Hydroxycoumarins
			163.0383	2.83	3-Hydroxycoumarin	Hydroxycoumarins
			189.0544	2.95	2,3-dihydrofuro[3,2-g]chromen-7-one	Psoralens
			197.1174	3.31	4-(3-hydroxybutyl)-2-methoxyphenol	Methoxyphenols
			371.1125	3.93	(7'S)-parabenzlactone	Dibenzylbutyrolactone lignans

		353.1025	3.96	3-(2,4-dihydroxyphenyl)-5-hydroxy- 8,8-dimethylpyrano[2,3-h]chromen- 4-one	Pyranoisoflavonoids
		207.0653	4.03	4-(2-Methoxyphenyl)-2-Oxobut-3- Enoic Acid	Cinnamic acids and derivatives
		311.0915	4.05	Pyranoxanthones	Pyranoxanthones
		329.1025	4.05	3,5-Dihydroxy-7-methoxyflavanone 3-acetate	7-O-methylated flavonoids
		313.1074	4.75	3-(3,4-dimethoxyphenyl)-7-methoxychromen-4-one	7-O-methylisoflavones
		191.0709	4.77	7-Methoxy-6-methyl-2H-1- benzopyran-2-one	Coumarins and derivatives
	Terpenes	349.2007	4.35	(1,5,8a-trimethyl-2,8-dioxo-3a,4,5,5a,9,9a-hexahydro-1H-azuleno[6,5-b]furan-9-yl) 2-methylbutanoate	Sesquiterpene lactones
		275.2012	5.36	(3E,5E,7E)-8-[(4R)-4-hydroxy-2,6,6-trimethylcyclohex-1-en-1-yl]-6-methylocta-3,5,7-trien-2-one	Sesquiterpenoids
		411.3623	7.10	(3S,10R,13R)-10,13-dimethyl-17-[(Z,2R)-5-propan-2-ylhept-5-en-2-yl] 2,3,4,9,11,12,14,15,16,17-decahydro-1H- cyclopenta[a]phenanthren-3-ol	Stigmastanes and derivatives
		457.3678	7.11	3,9-dihydroxy-4,6~{a},6~{b},8~{a},11,11,14~{b}-heptamethyl-1,2,3,4~{a},5,6,7,8,9,10,12,12~{a},14,14~{a}-tetradecahydropicene-4- carbaldehyde	Triterpenoids
		309.2790	8.11	13E-Labdene-8alpha,15-diol	Diterpenoids
		409.3831	11.69	Ferna-7,9(11)-diene	Triterpenoids
		409.3837	12.73	Sesterterpenoids	Sesterterpenoids
	Others	179.1070	3.00	(4S)-4-hydroxy-3-methyl-2-[(2Z)- penta-2,4-dienyl]cyclopent-2-en-1- one	Secondary alcohols
		181.1225	4.50	4-hydroxy-3-methyl-2-[(2E)-pent-2- en-1-yl]cyclopent-2-en-1-one	Secondary alcohols
		291.1961	5.17	Deoxy phytoprostane J1	Prostaglandins and related compounds
		293.2116	5.38	10-methoxyheptadec-1-en-4,6-diyne- 3,9-diol	Long-chain fatty alcohols
		221.1175	6.15	2-(1-hydroxycyclopentyl)-2- phenylacetic acid	Benzene and substituted derivatives

	279.2321	6.23	Octadeca-9,12,15-trienoic acid	Lineolic acids and derivatives
	256.2639	7.26	Hexadecanamide	Fatty amides
	265.2527	7.43	Octadeca-9,12-dienal	Fatty aldehydes
	613.4830	8.95	(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
	429.3732	9.26	2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chromen-6-ol	Tocopherols

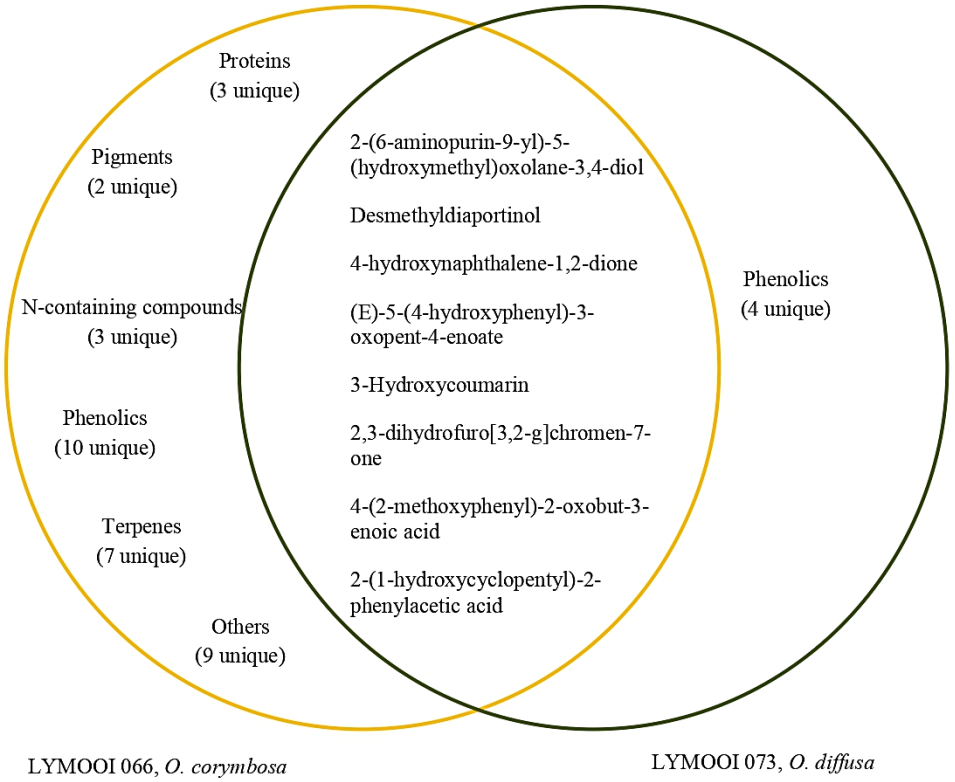

Figure 2. Venn diagram showing the common compounds that are shared between the *O. corymbosa* and *O. diffusa*

results of sample LYMOOI 073 can only be identified up to species level by using *mat*K but not by *rbc*L and ITS. Based on the results of this study, DNA barcoding did not distinguish closely related plant species and it may be due to insufficient sequence divergence in standard barcode markers [55-58]. There may be less suitable to use *rbc*L and ITS for identifying *O. diffusa.* According to Li et al. [59], *rbc*L sequence has evolved slowly, and this locus has the lowest divergence of plastid genes in flowering plants. On the other hand, it is reported that ITS regions usually undergo concerted evolution through unequal crossover, high-frequency gene conversion, and large deletions [60-62]. Hence, a more suitable plant barcode region should be found in the future for *O. diffusa* identification. Since the current DNA barcoding results were unable to identify *Oldenlandia diffusa* (Willd.) Roxb., the suggestion of Amandita et al. [63] can be applied by cross checking the result of sequence identification with morphological identification result. Thus, in this study, the identification of these plant species was conducted mainly based on morphological identification.

Non-targeted Metabolite Profiling

Plant therapeutic role is justified by the presence of their bioactive compounds [64]. In this study, the non-targeted metabolite profiling revealed several "known unknown" putative compounds by using MS-Finder software. A "known unknown" refers to compounds cited in chemical literature or mass spectrometry reference databases but compounds unknown to the researcher [65]. Majority of the metabolites content revealed for both *Oldenlandia* species were different, nevertheless there were 8 common putative compounds detected for both plants (Tables 5 and 6, and Figure 2). Besides, except for the four detected compounds that were known putative compounds (3-Hydroxycoumarin, 4-hydroxynaphthalene-1,2-dione, Octadeca- 9,12,15-trienoic acid, and Pyranoxanthones), the remaining were "known unknowns" putative compounds. The identified compound of 3-Hydroxycoumarin was previously reported as a potent human 15-LOX-1 inhibitor [66]; as an inhibitor for chitin synthase production by fungus [67]; as an inhibitor of tyrosinase [68], and as photoprotective drugs [69]. 4-hydroxynaphthalene-1,2-dione, a ligand of Lawsone [70] was detected and was previously reported to show antifungal activity. 3-Hydroxycoumarin and 4-hydroxynaphthalene-1,2-dione were detected in *O. diffusa* and *O. corymbosa*. Octadeca-9,12,15-trienoic acid shows anti-inflammatory and anti-atherogenic properties [71] was detected only in *O. corymbosa*. Pyranoxanthones were previously reported by Azevedo et al. [72] with the anticancer properties and it was detected in *O. corymbosa.*

The profiling analysis revealed the high contents of phenolic in both the plant samples. According to Maulidiani et al. [73], phenolic content is related to daylight with higher intensity (0% shade). Lighting conditions significantly affect the biosynthetic course of a growing plant. Correspondingly, the presence of triterpenoids, flavonoids, and phenolics compounds may act to protect the cells from injury by UV radiation [74] and prevent free radicals causing tissue damage by avoiding the formation of radicals, scavenging them or promoting their decomposition [75]. Growing condition of *Oldenlandia* under full light contributed to the high phenolic contents. Sunlight is absorbed by plants, producing high levels of oxygen and secondary compounds through photosynthesis; therefore, adverse environmental conditions may lead to high accumulation of phenolic contents in leaf epidermis [76, 77]. Phenolic compounds are the main class of secondary metabolites in plants and may contribute directly to antioxidative effects claimed for these two species [78-80]. As mentioned by Ji et al. [81], the antioxidant properties of *O. diffusa* make it as a potentially therapeutic herb for female-specific disorders including endometriosis and polycystic ovarian syndrome (PCOS). Besides, *O. corymbosa* has been reported [82] to have high amounts of flavonoid and phenolic (polyphenolic) compounds, which can be used to treat various oxidative stress-related diseases. Nevertheless, the function of phenolic compounds as antioxidants reported in this study remains to be determined, because most of them are considered as "known unknown".

CONCLUSION

In conclusion, complementarity of morphology and DNA barcodes have been successfully used to differentiate *O. diffusa* from *O. corymbosa*. This study indicates that morphological characteristics can identify both *Oldenlandia* species based on the size of leaves, number of flowers, and height of the plant. DNA barcoding analysis by using barcodes of *rbc*L*, mat*K, and ITS were able to identify *Oldenlandia corymbosa* up to species level, but not for *Oldenlandia diffusa*. Hence, searching for suitable loci to accurately identify *Oldenlandia diffusa* is essential as it is widely used as traditional Chinese medicinal herb. Non-targeted LC-MS/MS analysis was able to detect 12 putative compounds in *O. diffusa* and 42 putative compounds in *O. corymbosa*.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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