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COMPARATIVE PHARMACOGNOSTICAL AND UHPLC-QTOF MS ANALYSIS OF TWO SPECIES OF LILIUM

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ABSTRACT

Two species of Lilium, *Lilium lancifolium* Thunb. and *Lilium polyphyllum* D. Don both belonging to family Liliaceae, are used extensively in Traditional Chinese Medicine (TCM) and Ayurveda, respectively. In the current study, the tissue structure and metabolite differences between the bulbs of the two species were investigated by application of UHPLC-QTOF-MS and microscopy analysis. The study reveals that there are several morphological similarities between them. However, the secondary metabolite composition as identified in this study vary considerably. The study opens avenues for further detailed investigation of these species for their similarities and differences in therapeutic properties.

KEYWORDS: Lilium lancifolium, Lilium polyphyllum, Traditional

INTRODUCTION

The genus Lilium has about 100 species which are of ornamental and medicinal value, and are found in parts of Europe, China, India and North America.[1] The *Lilium lancifolium* Thunb. species is one of the 55 species of the genus Lilium that is used as an edible medicinal plant in Traditional Chinese Medicine (TCM). It is used for treating pneumonia, bronchitis, and as a rich source of antioxidants and steroidal saponins.[2–5] The bulbs of *L. lancifolium* are also reported to have anti-inflammatory and anti-bacterial properties, and alkaloids, saponins and phenolic compounds are the principle phytoconstituents of this plant.[6]

Lilium polyphyllum is the species extensively used in one of the traditional medicine systems of India called Ayurveda.[7] This species is reported to be found in North-west Himalayan region of India. The natural habitats of this plant have been extensively exploited to meet the market demands for supply of this drug.[8,9] This plant is now categorized as a critically endangered plant. In Ayurveda, this plant is used as an aphrodisiac, antipyretic, expectorant, and diuretic.[10,11]

The pharmacological properties of these two plants have been studied. But there is not an extensive literature available on their chemical profiling of these plants by application of hyphenated chromatography mass spectrometry methods. There is a need for extensive investigation of these two species to explore their medicinal uses further and knowledge of their phytochemical basis forms an important contribution to this exploration.

In this study we have studied the microscopical tissue characteristics of both these species and carried out the metabolite profiling of methanol extracts of the bulbs. The objective of this study is to suggest *L. lancifolium* as a qualitative substitute for *L. polyphyllum* which is a critically endangered medicinal plant species.

MATERIALS AND METHODS

Collection of plant material

The samples of the bulbs of *Lilium lancifolium* and *Lilium polyphyllum* were purchased from commercial herb markets from China and India, respectively. The samples were authenticated by Professor Zhongzhen Zhao, and voucher specimens were deposited in the Bank of China (Hong Kong) Chinese Medicines Centre of Hong Kong Baptist University, Hong Kong for both the species. The specimen voucher numbers obtained for *Lilium lancifolium and Lilium polyphyllum* were LLT-312 and LPB-123, respectively.

Microscopy and sample preparation

For studying the tissue structures of bulbs, sections of 20 µm thickness were prepared using a Cryotome (Thermo Shandon As620, UK). The samples were mounted on blocks of cryostats for sectioning by use of cryogel. The sections were mounted on glass slides for their light microscopy investigation. The tissue structures were investigated using a Leica DM5000 B light microscope (Leica Microsystems, Germany).

General experimental procedure

The profiling of metabolites was carried out using an Agilent 6540 accurate – mass Q-TOF LC/MS (Agilent Technologies, USA). The separation of metabolites was obtained using a UPLC C_{18} analytical column (dimensions: 2.1mm × 100 mm, I.D. 1.7µm, Waters, U.S.A.) and the analysis was performed at 20℃. The parameters used for MS analyses were as follows: temperature of dry gas was 300 ℃, flow rate of (N_2) gas was 6 L/min, the pressure of nebulizer was 40 psi, the applied fragmentor voltage was 150V, and the nozzle voltage was 500V. The acquisition of mass spectra was carried out in positive mode in the range 110 to 1700 *m/z*. A gradient elution was used for separation with mobile phase comprising of a mixture of H_2O(A) and CH_3CN (B), both containing 0.1% HCO_2H. The gradient elution was as follows: 0–10 min, B (5–25%); 10-25min, B (25-75 %); 25–28 min, B (75–100 %); 28-31 min, B (100–100 %); 31-31.1 min, B (100–5 %). The time used for equilibration was 2.9 min. the flow rate applied was 0.4 mL/min and the injection volume used was 2µL. ESI-low concentration tuning mix solution (Agilent technologies, USA) was used for accurate mass calibration. The mass error threshold was set to 10ppm and the collision energy was set to 20.0 and 40.0 volts. All the solvents used for mobile phase preparation were of HPLC grade and obtained from E.Merck (Darmstadt, Germany). Methanol was used a blank. Methanol was used as a solvent for extraction as it yielded the optimum elution and resolution of components in the samples. About 250 mg of each sample was extracted in 1.0 mL of methanol and used for elution. The samples were extracted in ultrasonic bath for 30 mins at room temperature. The samples were then centrifuged at (CREST 1875HTAG ultrasonic processor, USA) for 10 mins at 1000 rpm, and the supernatants were used for analysis. Triplicate samples of each selected species of Lilium tubers were prepared for analysis.

Data analysis

Identification of detected $[M+H]^+$ and $[M+Na]^+$ was carried out using Agilent Mass Hunter Workstation software-Qualitative Analysis (version B 4.00, Service Pack 3, Agilent Technologies, Inc. 2011). The settings applied for data analysis and identification were as follows: peaks with height ≥ 2000 counts, charge state of 1, and tolerance for peak spacing (0.0025 m/z, plus 7.0 ppm), extraction restricted retention time (1.0-25.0 min), absolute height ≥ 1500 counts, compound relative height ≥ 2.5 %. The peak list generated through Mass Hunter Workstation software was imported using Microsoft Excel software (Microsoft, Redmond,WA) for preparing peak list.

RESULTS

Morphological and microscopical analysis

The macroscopic structures of *L. lancifolium* and *L. polyphyllum* were found to be similar. The bulbs consisted of several layers of scales with marked longitudinal ridges. The scales were arranged in concentric manner. The size of scales of both the varieties were similar in lengths ranging from 2.0-3.0 cm and widths about 1.5-2.0 cm. Scars of adventitious roots were visible at the base of clusters of the scales. The sizes of the scales are observed to be reduced from the outermost layers to the innermost layers. The scales of *L. lancifolium* were found to be fleshier in appearance compared to *L. polyphyllum* (see **Fig.1**).

Fig. 1: Pictorial representation of scales of *L. lancifolium* (A) and *L. polyphyllum* (B).

The microscopical features of the scales of the bulbs in transverse sections were studied at 366 nm and in white light. The scales have an inner and an outer epidermis for both *L. lancifolium* and *L. polyphyllum*. The epidermis in the prepared sections of *L. lancifolium* was found to be thicker and darker in color compared to that of *L. polyphyllum.* The epidermis is made of a single layer of parenchymatous cells which are filled with mucilage and are pentagonal in shape. In 366 nm the epidermis does not exhibit any fluorescent colors different from the other cells. The cuticle of the epidermis appears wavy with groove like structures.

The mesophyll is made of several layers of parenchymatous cells filled with starch granules. The cells are hexagonal in shape and gelatinized. A few xylem vessels consisting of tracheid cell clusters are found scattered around the parenchymatous cells. The tracheid cells appear tubular in shape. It is observed that both the selected species of Lilium genus have similar tissue structures as indicated in **Fig.2 and 3**.

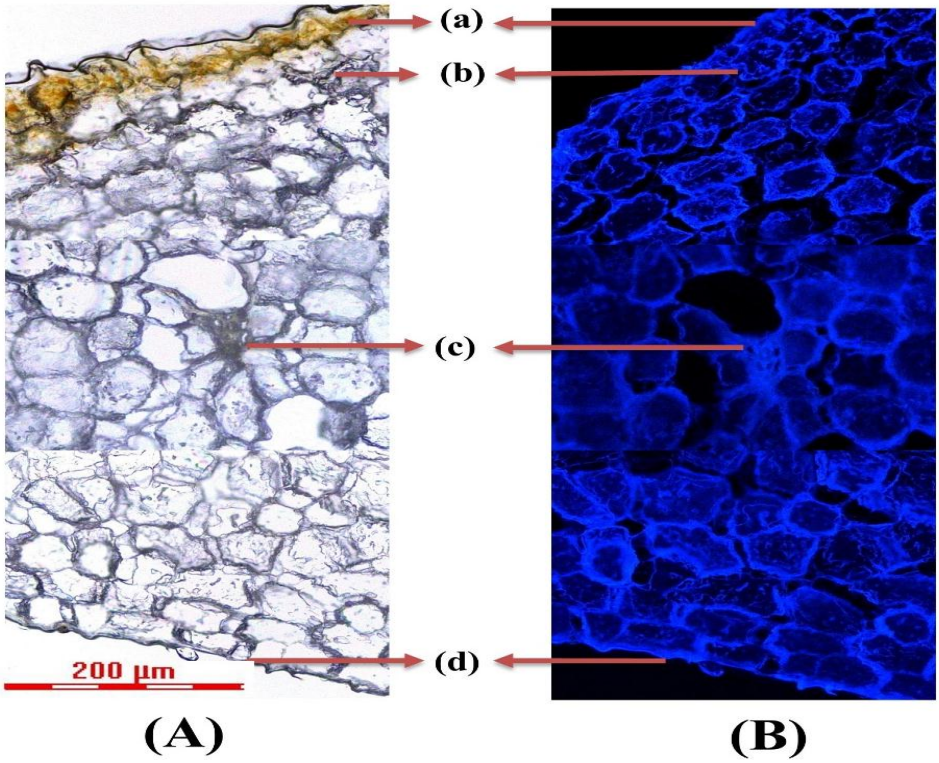

Fig. 2: Tissue structures of *L. lancifolium* studied under white light (A) and under 366 nm (B). (a) represents outer epidermis, (b) represents cortex cells, (c) represents xylem tracheid cells and (d) inner epidermis.

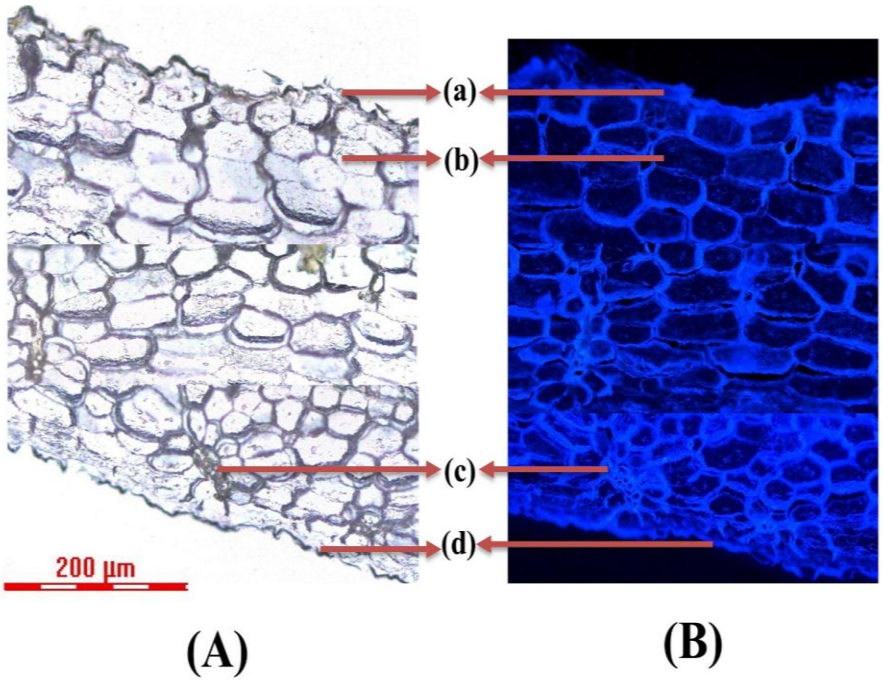

Fig. 3: Tissue structures of *L. polyphyllum* studied under white light (A) and under 366 nm (B). (a) represents outer epidermis, (b) represents cortex cells, (c) represents xylem tracheid cells and (d) inner epidermis.

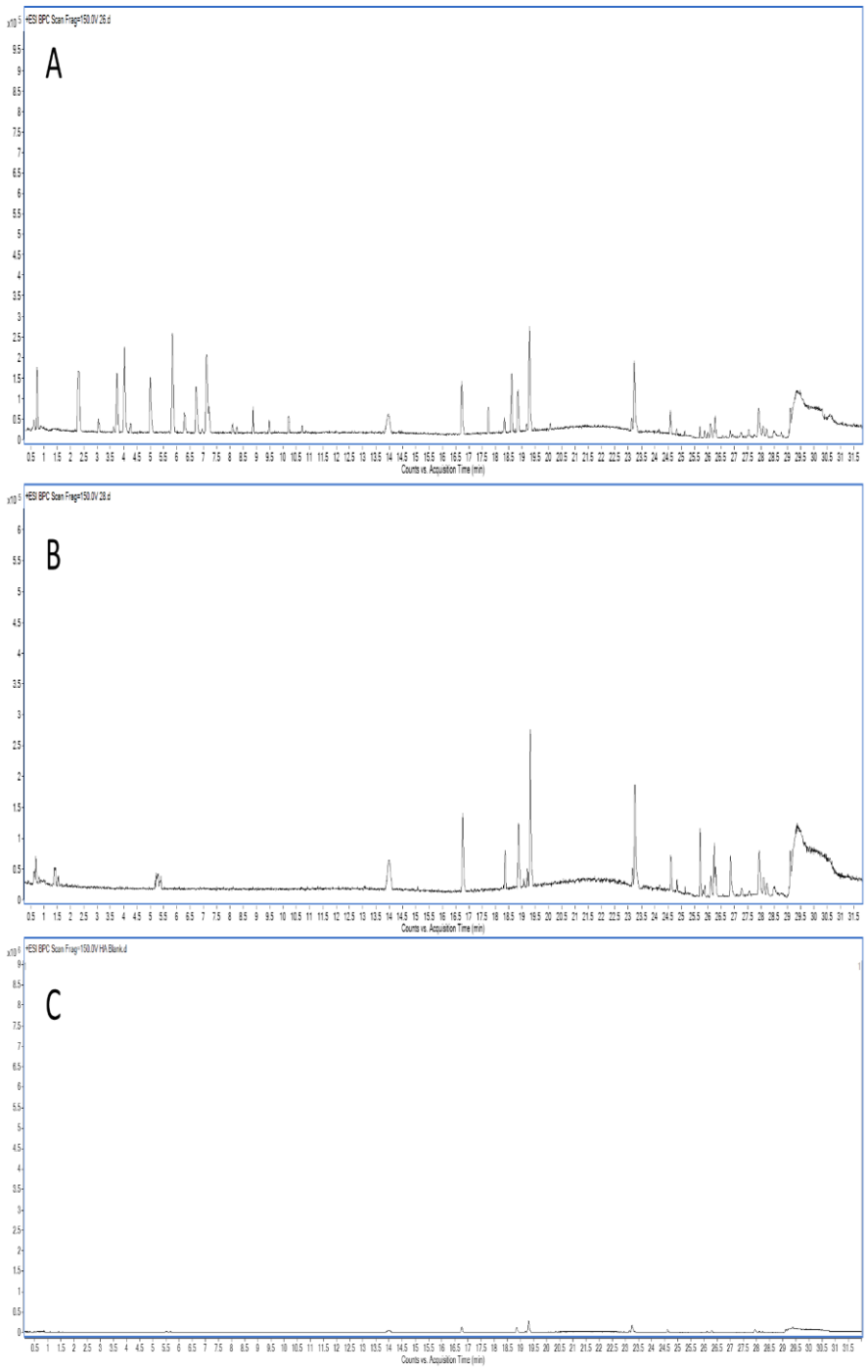

Fig. 4: Base peak Chromatograms (BPC) of extracts of bulbs of selected Lilium species (A) L. polyphyllum, (B) L. lancifolium and (C) Methanol used a blank.

LC-MS comparative metabolite profiling

The LC-MS analysis of the extracts of bulbs of both selected species of Lilium were prepared in methanol and analyzed in both positive and negative mode. The total number of peaks and the peak resolution were found to be better in positive mode, hence the profiling was carried out in positive mode. The base peak chromatograms of both the species are illustrated in **Fig.**

4. It was observed that with methanol as the extraction solvent the presence of phenolic glycosides, Regaloside A and B were found in the extracts of *L. lancifolium* species. This species also indicated the presence of Jatropham and coumaric acid derivatives including 1,3-O-diferuloylglycerol. The *L. polyphyllum* species indicated the presence of saponins and polyphenolic compounds as listed in **Table 1**. In total 12 constituents were identified through tentative identification in both the species in positive mode.

Table. 1: List of constituents tentatively identified by LC-MS analysis.

Peak nos.	Compound Name	Molecular Formula	Calculated Mass values	Rt	Species	Calculated m/z values	Observed m/z values	Δ ppm	Samples	
									CW	IW
1.	D-glucose	$C_6H_{12}O_6$	180.156	0.72	$(M+H)^+$	181.070	181.065	26.3	-	+
					$(M+Na)^+$	203.052	203.052	0.85	-	+
2.	p-coumaric acid	$C_9H_8O_3$	164.160	1.17	$(M+H)^+$	165.054	165.054	3.79	-	+
3.	Kaempferol 3,7-diglucoside	$C_{27}H_{30}O_{16}$	610.521	10.75	$(M+H)^-$	611.160	611.160	0.83	-	+
4.	Timosaponin A-I	$C_{33}H_{54}O_8$	578.787	12.25	$(M+H)^+$	579.389	579.388	1.85	-	+
5.	Timosaponin A III	$C_{39}H_{64}O_{13}$	740.928	12.33	$(M+H)^+$	741.442	741.441	1.09	-	+
6.	Pseudoprototimosaponin AIII	$C_{45}H_{74}O_{18}$	903.069	12.46	$(M+H)^+$	903.494	903.492	2.37	-	+
7.	Isoeugenol	$C_{10}H_{12}O_2$	164.204	17.90	$(M+H)^+$	165.091	165.091	-1.2	-	+
8.	Diosgenin	$C_{27}H_{42}O_3$	414.630	19.86	$(M+H)^+$	415.320	415.323	-6.97	-	+
9.	Jatropham	$C_5H_7NO_2$	113.116	0.79	$(M+H)^+$	114.055	114.055	-1.57	+	-
10.	Regaloside A	$C_{18}H_{24}O_{10}$	400.380	1.47	$(M+H)^+$	401.144	401.143	1.47	+	-
11.	Regaloside B	$C_{20}H_{26}O_{11}$	442.417	9.73	$(M+H)^+$	443.154	443.155	-0.85	+	-
12.	1,3-O-diferuloylglycerol	$C_{23}H_{24}O_9$	444.436	13.00	$(M+H)^+$	445.149	445.148	2.18	+	-

Footnote: Presence of the phytoconstituent is denoted by (+) and the absence is denoted by (-). **CW** and **IW** denote extracts of *L. lancifolium* and *L. polyphyllum*, respectively.

DISCUSSION

In this study, the tissue microscopical characteristics were compared for both *L. lancifolium* and *L. polyphyllum* species. Mass spectrometry and chromatographic profiling reveals presence of saponins, anti-oxidant polyphenols and other compounds which are responsible for the therapeutic effects reported in literature for these species. Further exploration with solvents of varying polarity used for extraction may reveal presence of other compounds in the bulbs of these two species. This study is a qualitative analysis study which may form basis for pharmacological studies to investigate and compare the therapeutic benefits of these species.

CONCLUSION

The study opens avenues for further exploration and comparison of the two selected species of Lilium genus, based on their pharmacological and phytochemical attributes. The results of the study serve as the basis to suggest L. lancifolium as a substitute to the endangered L. polyphyllum species.

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