Original Article

Pharmacognostical studies of *Premna microphylla*

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A R T I C L E   I N F O

A B S T R A C T

Various traditional systems of medicine enlightened the importance of *Premna microphylla* Turcz., Lamiaceae, medicinally. The present study was carried out to provide a scientific basis of the identification and the authenticity of *P. microphylla* with the help of pharmacognostical parameters, which is not done before. Roots, stems, and leaves of *P. microphylla* were collected for pharmacognostical studies involving macros, microscopic evaluation, physicochemical parameters analysis like fluorescence analysis and thin layer chromatography, in addition with DNA barcodes of internal transcribed spacer and *psb*A-*trn*H regions. Transverse section of root indicated the presence of stone cell bands. Transverse section of stem showed the presence of stone cells and vessels. Transverse section of leaf midrib revealed the presence of shaft type of porosity. Microscopic studies of powder revealed the presence of cork cells, fibers, vessels, nonglandular hairs, stone cells and glandular scale cells. Thin layer chromatography of the extract revealed the presence of oleanolic acid in *P. microphylla* with specific R_f values. Identification through DNA barcode showed the sequence of internal transcribed spacer region was novel while the sequence of *psb*A-*trn*H region displayed no differences from known sequence. The observations confirmed that *P. microphylla* has an obvious pharmacognostical characteristics, which will be useful toward providing a reliable basis for identification, purity, quality and classification of the plant.

Introduction

Premna microphylla Turcz., a medicinal and edible plant locally known as "doufuchai", belonging to the family Lamiaceae, is a perennial deciduous shrub with a height of 2–6 m tall, which widely distributes in tropical and subtropical regions of the world such as Asia, Australia and Africa, and in China. It is broadly distributed in the mountainous regions in the east, middle, and south. Normally it grows up in acid soil environment where the altitude lies from sea level to 500–1000 m (Zhang et al., 2017). The leaves, blade is ovate-lanceolate, elliptic, ovate, or obovate in shape, measuring 3–13 cm long and 1.5–6 cm wide. The apex is long acuminate to acute, and based part is narrowly cuneate with margin entire or lobed to sometimes serrulate. The petiole ranges the length from 0.5 to 2 cm long. Inflorescences are in the form of conical panicles. Fruit is purple in color with globose to obovate in shape. The seeds, with 1000-grain weight about 17.5 g, are difficult to breed though the setting rate is high.

Premna microphylla has been highlighted the use of its roots, stems and leaves as Traditional Chinese Medicine (TCM) for the treatment of numerous ailments like skin burning and bleeding,

rheumatism, dysentery, swelling and viper bites (Chen et al., 2014). While the antioxidant, antimicrobial and cytotoxic activities have been found in essential oil (Zhang et al., 2017), leaves and stems (Xu et al., 2010) from this plant. In addition, the extracts of its leaves which can be used to prepare "green tofu" by local people for its high amount of pectin, have been used to treat fatigue and inflammation (Chen et al., 2014).

The chemical composition and efficacy of extracts from *P. microphylla* were intensively investigated in previous studies. Two new xanthones (Wang and Xu, 2003), four new isoflavones (Zhong and Wang, 2002), a new triterpene glycoside (Zhan et al., 2009) as well as two new glyceroglycolipid and ceramide (Zhan and Yue, 2003) from the extracts of this plant have been isolated and their structures elucidated. In addition, fifty-six compounds were identified in the essential oil of *P. microphylla* (Zhang et al., 2017). The pectin from *P. microphylla* leaves has been extracted for analyzing the cell wall composition, observing the morphology of residues after each extraction steps and presenting physicochemical properties of different pectic substances (Chen et al., 2014). The complete nucleotide sequence of the *P. microphylla* chloroplast (cp) genome was reported and characterized (Yang and Kong, 2016). As for the pharmacognostical studies, the anatomic structure of the stems and leaves of *P. microphylla* have been reported (He et al., 2011), which was the only one report involving the pharmacognostical studies of it so far but it was not complete and systematic. Therefore, the

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information regarding its pharmacognostical identification on the roots, stems and leaves is still very scanty and poor understood.

As the drug is endowed with huge exploitation and utilization value, it is medicinally important to know precisely and comprehensively about its characteristics of pharmacognosy. With some supplementary in the previous study on plant anatomy of *P. microphylla* (He et al., 2011), herein we made a detailed investigation on macroscopic, microscopic characters, histochemistry, physicochemical parameters, fluorescence analysis, powder behavior and DNA barcodes of this plant to help in its identification and standardization.

Materials and methods

Collection and identification of plant material

Fresh plants of *Premna microphylla* Turcz., Lamiaceae, were collected from Guangdong Pharmaceutical University, Guangzhou Higher Education Mega Center (23°3′39″ N 113°23′54″ E), identified and authenticated by Prof. Shengguo Ji, School of Chinese Traditional Medicine, Guangdong Pharmaceutical University. They were washed and cleaned by flowing water to remove the physical impurities, air-dried in the shade, made into powder in a blender and preserved in hermetic container with dry air for pharmacognostical study.

Preparation of sample

Transverse section

Transverse sections of fresh roots, stems and leaves of *P. microphylla* were made by hand, which were immobilized in FAA solution (formalin:glacial acetic acid:70% ethyl alcohol; [5:5:90]) for macro- and microscopic observations (Johansen, 1940).

Leaf epidermis

Fresh leaves of plant were subjected to obtain the upper and lower epidermis by tweezers, put into a petri dish with distilled water and cut into slices to be observed of cells and stoma. The stomatal index is calculated by the following formula (Kang, 2005).

$$\% \text{ of Stomatal Indices} = \frac{\text{Stomatal number per unit area}}{\text{Stomatal number per unit area} \times \text{Epidermal cell number of same area}} \times 100\% (1)$$

Powders

The powder was separately treated with glycerine (50%, v/v) and chloral hydrates (10%, v/v) through the heating for microscopic study.

Macroscopic characters

The organoleptic and morphological characters of fresh material including color, shape, size, texture and fracture were studied and noted.

Microscopic characters

The powders of plant were studied microscopically and fresh material fixed with the FAA was used for histologic study. Sliced by paraffin section method, the thin hand cut sections of roots, stems and leaves were dehydrated in a series alcohol concentration, followed by staining with safranine-fast green, mounting with neutral resin (Johansen, 1940). Microphotographs were taken by observing the free hand sections under Motic Multi-plexer attached to the microscope. All important features were detected and recorded suitably.

Physicochemical parameters analysis

The physicochemical parameters analysis of the powders, including behavior of powder drug, fluorescence and TLC analysis were determined as per the standard guidelines (Kokate, 1998; WHO, 1998; Khandelwal, 2001).

Behavior of powders

The powders were treated with different regents namely glacial acetic acid, sulfuric acid, hydrochloric acid, nitric acid, ferric chloride, sodium hydroxide and potassium hydroxide. The behavior of powders like floating or sinking and changes of solution colors were observed.

Fluorescence analysis

Fluorescence analysis was carried out by ultrasonic processing the powdered drug with different reagents, namely ethanol (75% v/v), ethyl acetate, acetone, methanol, chloroform, carbon tetrachloride, water and petroleum ether and observed at 254 nm, 366 nm in a UV chamber and visible light.

Thin layer chromatography

Thin layer chromatography studies were carried out for methanol extract of *P. microphylla* and reference sample oleanolic acid. The spots obtained from both the extracts were examined under visible light. An aluminum plate (20 cm × 10 cm) precoated with CMC-Na (0.5%)-silica gel G was used as the absorbent. The solvent system was toluene-ethyl acetate-glacial acetic acid (12:4:0.5). The methanol extract of *P. microphylla* was prepared by using 2 g powder, ultrasonic treated with 50 ml of methanol, filtered and evaporated to dryness. The plate was developed in a Camag twin trough chamber at temperature of 14 °C and relative humidity 69%, and sprayed with 10% ethanol sulfuric acid for coloration, dried at 105 °C and examined at visible light.

Identification through DNA barcode

Extraction of genomic DNA

The samples were cleaned with pure water, followed by being scrubbed with ethanol (75%, v/v) and placed in a mortar adding liquid nitrogen to be quick-frozen, and made into coarse powder (Marieschi et al., 2012). The total DNA was extracted by Plant DNA Extraction Kit (Guangzhou Xueyou Biotechnology Co. Ltd., China, Batch number: 2017011537) following manufacturer's instruction, and its purity was checked using Ultraviolet Micro-Spectrophotometer (K5500Plus, Beijing Kaiao Technology Development Co. Ltd., China).

Amplification of internal transcribed spacer region

Complete internal transcribed spacer (ITS) region of *P. microphylla* was amplified with the universal primers ITS4 (forward primer; 5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (reverse primer; 5′-TCCTCCGCTTATTGATATGC-3′) (Balasubramani et al., 2011a). The primers were custom synthesized by Shenzhen Huada Gene Technology Co. LTD. (Shenzhen, China). Amplification was carried out in 20 μl reaction volume with 5.9–8.6 μl sterile double-distilled

water, 0.2–1.0 µl forward and reverse primer, 1.0–2.5 µl Template DNA and 10 µl Master Mix. The amplification profile was 94 °C for 5 min followed by 34 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s with a final extension step at 72 °C for 10 min.

Amplification of psbA-trnH *region*

Part of *psbA-trnH* region of *P. microphylla* was amplified with the universal primers p-Z2 (forward primer; 5′-GTTATGCATGAACGTAATGCTC-3′) and p-F2 (reverse primer; 5′-CGCGCATGGTGGATTCACAATCC-3′). The primers were custom synthesized by Shenzhen Huada Gene Technology Co. LTD. (Shenzhen, China). Amplification was carried out in 20 µl reaction volume with 5.9–7.7 µl sterile double-distilled water, 0.4–1.0 µl forward and reverse primer, 1.0–2.5 µl Template DNA and 10 µl Master Mix. The amplification profile was 94 °C for 5 min followed by 34 cycles of 94 °C for 30 s, 61 °C for 30 s, 72 °C for 60 s with a final extension step at 72 °C for 10 min.

Direct sequencing of ITS and psbA-trnH *region*

PCR products were resolved on 2% agarose, 1 × TAE gels. 6 µl PCR products was loaded to an Electrophoresis System (100 V constant pressure, DL-600C, Beijing Donglin Changsheng Biotechnology Co. Ltd., China) for 30 min, and was visualized under Gel Imaging Analysis System (Shanghai Lincheng Biotechnology Co. Ltd., China). The complete sequences of the ITS and *psbA-trnH* region for *P. microphylla* studied were then deposited at GenBank (http://www.ncbi.nlm.nih.gov/).

Results

Macroscopic characters

Fresh plant

Premna microphylla is a up-right shrub with a height of 2–6 meters tall. The branchlets are pubescent when young, while glabrescent when old. Leaves are ovate-lanceolate, elliptic, ovate or obovate in shape measuring 3–13 × 1.5–6 cm with bad smell when being rubbed. The inflorescence is cymose to paniculiform in terminal tower shape. The calyx with green or slightly purplish color is pubescent or glabrescent with cup-shaped whereas the edge is subequally 5-dentate with eyelashes commonly. Corolla is outside puberulent and glandular with slightly yellowish color whereas inside pilose especially at throat. Fruit is purplish in color with globose to obovate in shape (Fig. 1A).

Dried plant

Stems are yellowish green in color, easy to break up by hand. The young shoots are pilose, while the old branches are nearly glabrous. Opposite to each other with petiole measuring 0.5–2 cm long, leaves are ovate-lanceolate, obovate or oval in shape and green color with bad smell, measuring 3–13 cm long and 1.5–6 cm wide with narrow base and entire margin or irregular coarse teeth, apex acute to acuminate which is glabrous or pubescent (Fig. 1B).

Microscopic characters

Root

Transverse section of root is circular in outline consisting of epidermis, cortex and vascular zone. Cork layer is relatively thick in 4–6 layers of cells with scattered stone cell groups and phellogen is formed into a ring. Cortex is narrow with stone cells scattered in thin walled parenchyma. Phloem is thin with scattered stone cell groups composed of 2–4 cells and cambium is formed into a ring. Xylem is well-developed and wider measuring about three-fourths of the diameter of the cross section with singly disperse or

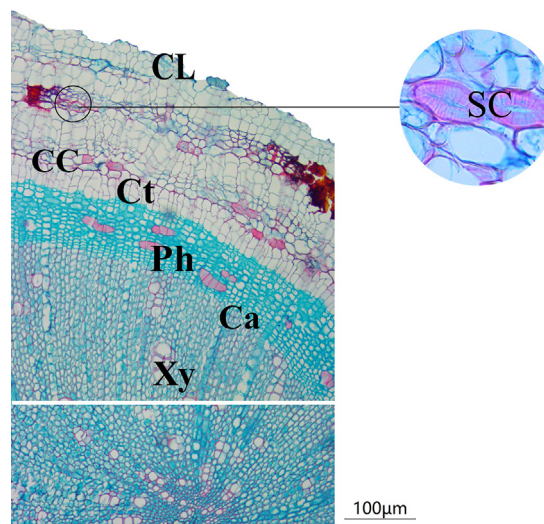**Fig. 1.** Fresh (A) and dried plant (B) of *Premna microphylla*.

Fig. 2. Transverse section of root of *Premna microphylla*. CL, cork layer; SC, stone cells; CC, cork cambium; Ct, cortex; Ph, phloem; Ca, cambium; Xy, xylem.

2–3 gathered vessels. Xylem rays are conspicuous, made up of 1–2 layers of cells (Fig. 2).

Stem

Transverse section of stem is quasi-circular in outline consisting of epidermis, cortex and vascular zone. Epidermis is made up of one layer of quasi-square cells arranged densely surrounded by cuticle. Cortex is made up of parenchymatous cells in quasi-circular or quasi-elliptic shaped with diffuse stone cells filled with reddish-brown stuffs in intercellular cavity. Phloem is narrow arranged around xylem, which is well-developed and strongly lignified dif-
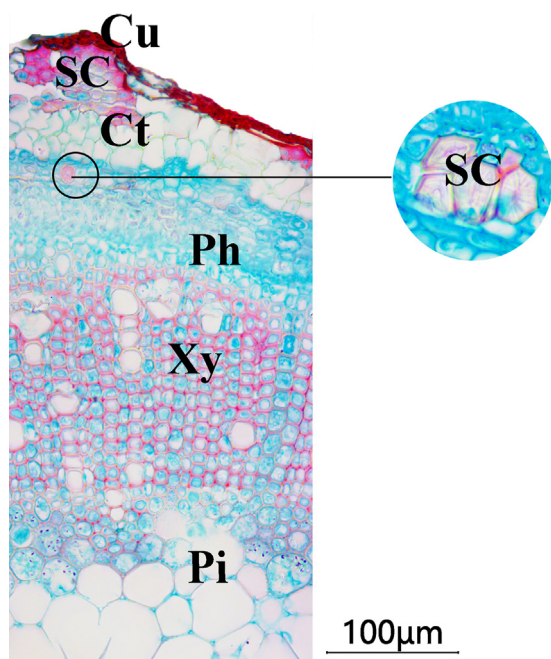
Fig. 3. Transverse section of stem of *Premna microphylla*. Cu, cuticle; SC, stone cells; Ct, cortex; Ph, phloem; Xy, xylem; Pi, pith.
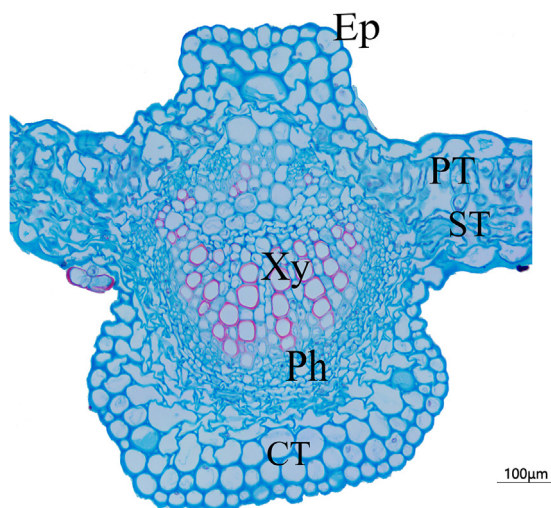

Fig. 4. Transverse section of leaf mibrib of *Premna microphylla*. Ep, epidermis; PT, palisade tissue; ST, spongy tissue; Xy, xylem; Ph, phloem; CT, collenchyma tissue.

fuse vessels. At center, pith is slightly hollow with disrupted cells (Fig. 3).

Leaf midrib

The epidermis consists of a layer of cells in quasi-circular or quasi-square shape covered by cuticle. There are 1–2 layers of palisade tissue of mesophyll under the upper epidermis. Spongy tissue is loosely arranged with bigger gap. Xylem is arranged diffusely in a ring and lignified surrounded by narrow phloem. Inside the lower epidermis, there are layers of collenchymas with bigger cells (Fig. 4).

Epidermis

Both the upper (Fig. 5A) and lower (Fig. 5B) epidermis cells are irregular-shaped form with anticlinal wall in wavy shape and clearly horny grains. The lower epidermis has stomatas, which are

Table 1
Behavior of powders of *Premna microphylla*.

Regent	Solution color	Behavior of powders
Glacial acetic acid	Green	Floating
Sulfuric acid	Greenish brown	Charry
Potassium hydroxide	Green	Floating
Sodium hydroxide	Green	Floating
Ferric trichloride	Yellowish green	Floating
Nitric acid	Brown	Floating
Hydrochloric acid	Green	Floating

Table 2
Fluorescence analysis of *Premna microphylla*.

Reagent	Visible/day light	UV 254 nm	UV 366 nm
Water	Brown	None	None
Ethanol (75%)	Brown	Orange	None
Ethyl acetate	Brown	Pinkish red	Pinkish red
Acetone	Yellow	Pinkish red	Pinkish red
Methanol	Brown	Pinkish red	None
Chloroform	Yellowish brown	None	None
Petroleum ether	Brown	None	None
Carbon tetrachloride	Brown	None	None

diacyctictype or infinitive, surrounded by two to four subsidiaries with stomatal indices at 12.5% and rare eight glandular scale cells.

Powders

Microscopic study of powder (Fig. 6) reveals the presence of cork cells, fibers, vessels, nonglandular hairs, stone cells and glandular scale cells. Cork cells are yellowish brown in color and quasi-square in shape with thicker wall and clearly horny grains. Fibers are yellowish green colored and lank shaped with a diameter of 20–40 μm long and thicker wall lignifiedly, obvious perforated orifice and large intercellular cavity. Most of vessels are spiral or bordered pit shaped ranges with a diameter of 31.2–56.4 μm long. The pits are closely arranged with oval aperture. Nonglandular hairs made up of many cells are curly and pyamhorm in shape with thick wall and obvious intercellular cavity. Stone cells, scattered singly or formed in groups, measuring about 65–110 μm, are atrovirens colored and quasi-rectangle shaped with thicker wall, narrow intercellular cavity and obvious aperture and colporate. Glandular scale is made up of eight cells with oblate head and quasi-round in top surface, and adenophore is shorter made up of one cell.

Behavior of powders

Powder analysis showed different appearances and solution colors treating with different reagents (Table 1).

Fluorescence analysis

Fluorescence analysis of powder and different extracts of *P. microphylla* with different reagents were carried out to observe the color reactions (Table 2).

Thin layer chromatography

The TLC profiles of *P. microphylla* extract and oleanolic acid standard were obtained under visible light. Distinct TLC spot on the silica gel plate representing oleanolic acid with specific R_f 0.64).

Identification through DNA barcode

Direct sequencing of the gel purified amplicon yielded a 698 bp sequence of ITS region and a 427 bp sequence of *psb*A-*trn*H region for *P. microphylla* (Table 3). The sequence of ITS (Fig. 7) region was deposited into GenBank (accession numbers MG991100) and the
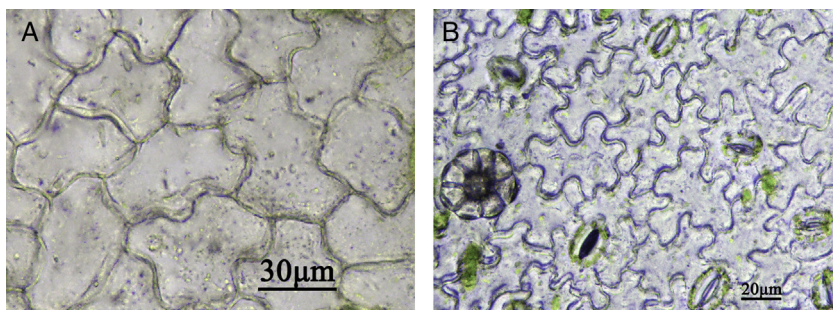
Fig. 5. Epidermis of leaf of *Premna microphylla*. (A) Upper epidermis; (B) lower epidermis.
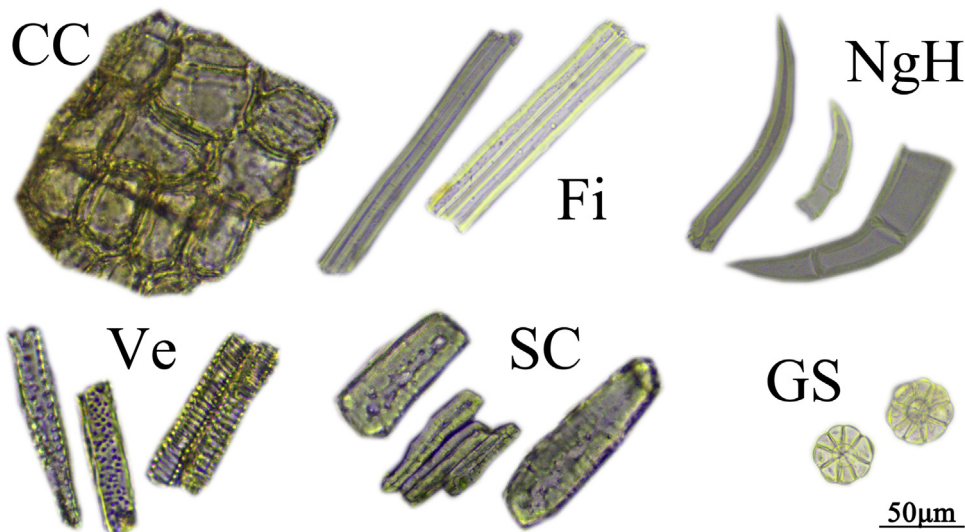

Fig. 6. Powder microscopy of *Premna microphylla*. CC, cork cells; Fi, fibers; NgH, non-glandualar hair; Ve, vessels; SC, stone cells; GS, glandular scale.

Table 3
Amplified sequences of ITS and *psb*A-*trn*H region for *Premna microphylla*.

Region	Primer	Sequence (5′-3′)	Base content (%)				Amplicon length (bp)	GenBank accession no.
			A	C	G	T		
ITS	Forward: ITS4 Reverse: ITS5	TCCTCCGCTTATTGATATGC TCCTCCGCTTATTGATATGC	21.2	26.1	27.8	24.9	698	MG991100.1
*psb*A-*trn*H	Forward: p-Z2 Reverse: p-F2	GTTATGCATGAACGTAATGCTC CGCGCATGGTGGATTCACAATCC	38.6	14.8	14.3	32.3	427	HQ427024.1

BLAST of its nucleotide sequence turned to be novel and did not have homology with any coding sequences of structural genes, while the nucleotide sequence of *psb*A-*trn*H region revealed no differences from that has been isolated before by Pei (accession numbers HQ427024.1) (Pei et al., 2011).

Discussion

The standardization of a herbal medicine is of key importance in establishing its proper identity, also plays an imperative role in understanding its structure, biology, botanical quality and clinical efficacy due to the often finding of substitute or counterfeit herbal materials in the market (Akbar et al., 2014). The analysis of macroscopic and microscopic characters, chemical and physical parameters and genetic information are the confirmatory tests for standardization. Thus, it is essential to study pharmacognostical characters of a medicinal plant. However, the plant *P. microphylla* is not yet having a much proven evidences for its standardization in modern system of medicine despite of its widely uses among local people for the treatment of various disease, as seldom pharmacognostical or anatomical work is on record to justify its authenticity. Considering these facts, authors have made an attempt to finger out pharmacognostical characters that can be utilitarian in establishing the identity and standardization of this plant.

The study provided some basic data regarding the genuine crude drug. The macroscopic properties including taste, sight, smell and touch were observed to give a primary indication about quality variation. The prominent diagnostic features of microscopy were cork cells, fibers, vessels, nonglandular hairs, stone cells and glandular scale cells, which can be considered as distinguishing characteristic for determining the anatomical structures and setting up the correct identity of this plant.

Fluorescence analysis is a necessary parameter that is unique to the plant and indicates the sign of chormophore in the drug, which is required in its first line standardization (Prasanth et al., 2017). Some plant constituents show fluorescence in the ultraviolet or visible light because they may often be converted into

tttggaaagaaaaaaatcgtaacaaggttttcgtaggtgaaccggtagaaggatcattgt	60
caaaacttgcaaagcagaccgtgaacacgtgtttaacatcaggaggccgcgatgcgggg	120
tgagccttccactgtagcacccatccccgtcggcgctcgctcgagtgtcgcatcgtatgg	180
gctaacaaactcgtgcgtgaaatcactaagaaaaactcagcgaagtgttcccccacctga	240
cgcgccgtccacagggcatgcgaggagaaagggatgtctctcgaatgtcaaaacgactct	300
cagcaacgaatatctcggctctcacattgatgaagaacgtaacaaaatgctatacttggt	360
gtgaattgccgaatgtcgtgaactatcgagtctttgaacacaagttgcgctcgaggccat	420
taggcccagggcacgtctacttgggcatcacgtattgcgtcgcccccctccggcactcct	480
cacaggcgagggggcggagattggcctcccatgtgcgtccgtgtgcggccggcctaaat	540
cccatccctcagcgacgcacatcacggccagtggtggttgaacaatcaattctcttgctg	600
tcgtgcaacaaaggcgttgcccgttcgagagctaaatgcgacccacgatgcttgctgcaa	660
agcacctctgaccgcgaccccagtcagtagatttccct	698

Fig. 7. ITS sequence of *Premna microphylla*.

fluorescent derivatives by applying different regents, which is very helpful to distinguish from suspicious specimens as extremely fast techniques.

TLC analysis serves as an important and powerful tool for standardization and determination bioactive compounds. The plant extract represented the distinctive TLC spots that similar to the R_f values of oleanolic acid, which indicated that the content of oleanolic acid was one of the test indexes in determining bioactive compounds of this plant.

DNA barcoding opens up a unique avenue for authentication and identification of herbal materials. The most suitable region of DNA barcodes applying in herbal materials is ITS2 region as a novel universal barcode as well as *psb*A-*trn*H region as a complementary, which is suggested to have great usability and variation by combination of both (Zhang et al., 2014). In this study, both ITS2 and *psb*A-*trn*H regions were used as a molecular pharmacognostical tool to identify *P. microphylla*, which gave confirmed result or clue for the above traditional methods. DNA information is currently considered to be rather reliable enough for identification of herbal materials, but one disadvantage is that it is not directly correlated with the contents of the active principle or chemical constituents (Balasubramani et al., 2011b). Therefore, it is critical to combine traditional methods with DNA information for systematic and complete identification of herbal materials.

In general, information generated in this work may be useful to further development of *P. microphylla*, which may act as reference information and produce a solid basis for proper identification, authentication, collection and investigation of the plant material. Further, it will be helpful to fetch the attention of pharmacologist to explore this plant in the line of scientific research.

Funding

Authors' contributions

GQ Tang, XJ Lin, JN Li and R Li assisted in carrying out the laboratory work. GQ Tang collected the plant material, worked on laboratorial tests, as well as wrote and formatted the paper. XJ Lin performed the scanning electron microscopy analysis and assisted with writing. D Wang and SG Ji created the project, supervised the study, the writing and review of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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