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PHARMACOGNOSTIC STUDIES OF *MORINDA BREVIPES* S. Y. HU

RONG LI, XIAOJING LIN, GENGQIU TANG, JUNNI LI, DONG WANG AND SHENGGUO JI*

School of Traditional Chinese Medicine, Guangdong Pharmaceutical University, Guangdong 510 006, PR China

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Abstract

DNA barcoding of ITS and psbA-trnH regions, histochemistry as well as thin layer chromatography (TLC) of *Morinda brevipes* S.Y. Hu were analyzed. Transverse section of root revealed the presence of cortex, xylem, cork cell, stone cells, and calcium oxalate sandy crystal. The lower epidermis cells showed many stoma in paracytic or inequality type. Spiral vessel and tiny calcium oxalate needle crystal usually appeared in the powder. TLC showed the presence of emodin in *M. brevipes*. Phytochemical studies revealed the existence of carbohydrates, saponins, tannins, flavones, anthraquinones, alkaloids and volatile oils. The ITS and *psb*A-*trn*H sequences were found for the first time which were submitted to NCBI to obtain the GenBank registration number. This study might play an important role in the identification, and utilization of *M. brevipes* for various purposes.

Introduction

It was reported that about 40,000 plants are used across the world to treat various ailments. In the last few decades, pharmaceutical manufacturer companies have focused on research and development of newly occurring plant-derived drugs. The macroscopic and microscopic descriptions of a medicinal plant are very important step for authentification of such plants (Ilgun *et al*. 2016, Blainskil *et al*. 2017). The DNA barcoding has become very important for identifying traditional Chinese medicine (TCM) (Chen *et al*. 2010, Dezhu *et al*. 2011, Li *et al*. 2011, Chen *et al*. 2014, Echen *et al*. 2014). In this regard, the ITS (Chase *et al*. 2005) and psbA-trnH (Kress and Erickson 2007) sequences can be mentioned for confirming the indentification of plants. TCM is becoming more and more popular and has gained scientific interest, which led to the introduction of TCM herbal drug monographs in the European Pharmacopoeia (Huang *et al*. 2014, Mei and Franz 2015). *M. brevipes*, belonging to the genus *Morinda*, family Rubiaceae, is a liane which principally distributes in Hainan province of China (Zhengyi 1999). Normally it grows up in hilly and mountainous regions and climbs or winds on a shrub or tree trunk where the altitude lies from sea level to 200 - 800 meters. As a TCM, *M. brevipes* has been used for the treatment of cold, cough, bronchitis, pertussis, diarrhea, bruises, strain of lumbar muscles and eczema (Zhengyi 1999). Since *M. brevipes* is endowed with huge exploitation value, it is important to know about its characteristics of pharmacognosy precisely and comprehensively. At present, there is no report on the study of traditional pharmacology and DNA barcoding of *M. brevipes*. Therefore, the lack of systematic identification method might lead to the confusion of cultivation and utilization of its varieties and the trouble of developing products. In this regard, DNA barcoding, macroscopic, microscopic characters, and powder analysis of plants were studied comprehensively.

Materials and Methods

Fresh plants were collected from Guangzhou Higher Education Mega Center (23°3′39″N 113°23′54″E), and identified as *M. brevipes* of genus *Morinda*, family Rubiaceae by Shengguo Ji, School of Traditional Chinese Medicine, Guangdong Pharmaceutical University. They were washed by flowing water to remove the physical impurities, air-dried under shade and made into a coarse powder using a mechanical blender for pharmacognostic study.

*Author for correspondence: <shengguo_ji@163.com>.

The macroscopic and morphological characters of fresh material including color, shape and size were observed in the laboratory. Microphotographs were taken by Motic Multi-plexer attached to the Motic-BA310 digital microscope. The root, stem, and leaf of fresh material were cut to the right size and were soaked in FAA fixative solution for more than 24 hrs. Afterwards, they were dehydrated in a series alcohol concentration and were made into microslide with paraffin section method (Johnson 1940). They were stained with safranine-fast green and mounted with neutral resin. Finally, they were prepared for paraffin sections which were observed under microscope.

The leaf was torn to thin pieces to get the upper and lower leaf epidermis by using tweezers which were put into a vessel containing distilled water. A suitable blade was chosen to make into microslide and was placed under a microscope. The root, stem, and leaf of dried material were crushed into powder by shredding machines which were sieved through 60 mesh sieves. They were treated with glycerine (50%, v/v) and chloral hydrates (10%, v/v) (Roy *et al*. 2016).

The 5 g powder of medicinal materials were extracted with 30 ml water, 30 ml petroleum ether, 30 ml 75% ethanol and 30 ml 0.5% hydrochloric acid solution by ultrasonic extractor for 30 min, respectively. The extracts were inspected by characteristic reaction (Kamble *et al*. 2017).

The powder (2 g) was extracted with 20 ml ethanol (95% v/v) by ultrasonic cleaner for 30 min. Based on previous studies, it was found that emodin is one of the main chemical constituents of the plant which could be separated by a specific thin-layer method. The extract and emodin reference samples were chromatographed on an aluminum plate (10 × 10 cm) which was precoated with CMC-Na (0.5%)-silica gel G (Qingdao Ocean Chemical Co. Ltd., China, Batch number: 20171010). The solvent system was toluene: ethyl acetate: formic acid (8 : 2 : 0.1, v/v/v). The plate was sprayed with sulfuric acid ethanol (10%) for coloration, dried at 105ºC and examined at visible light.

A proper amount of liquid nitrogen was added to the freeze fresh plants which were fully ground to powder. DNA of plants was extracted by using DNA extraction kit (Guangzhou Xueyou Biotechnology Co. Ltd., China, Batch number: B002006017). The whole ITS fragment and *psb*A-*trn*H fragment were amplified by PCR of which amplification conditions are shown in Table 1. Forward primer (0.6 µl), reverse primer (0.6 µl), DNA template (2 µl), ddH_2O (6.8 µl), Master Mix (10 µl) were put into the reaction system of ITS and *psb*A- *trn*H fragment. The PCR products (6 µl) were detected by agarose gel electrophoresis for 30 min. The concentration of agarose gel was 0.15% for ITS fragment and was 0.2% for *psb*A- *trn*H fragment.

Table 1. PCR amplification conditions.

Primer	Designation	Primer sequence (5' - 3')	Reaction time
ITS	ITS-F	TCCTCCGCTTATTGATATGC	94ºC, 5 min; 94ºC, 30 s; 54ºC, 30 s;
	ITS-R	GGAAGTAAAAGTCGTAACAAGG	72ºC, 1 min, 34 cycles, 72ºC, 10 min
*psb*A-*trn*H	P-F	GTTATGCATGAACGTAATGCTC	94ºC, 5 min; 94ºC, 30 s; 61ºC, 30 s;
	P-R	CGCGCATGGTGGATTCACAATCC	72ºC, 1 min; 34 cycles, 72ºC,10 min

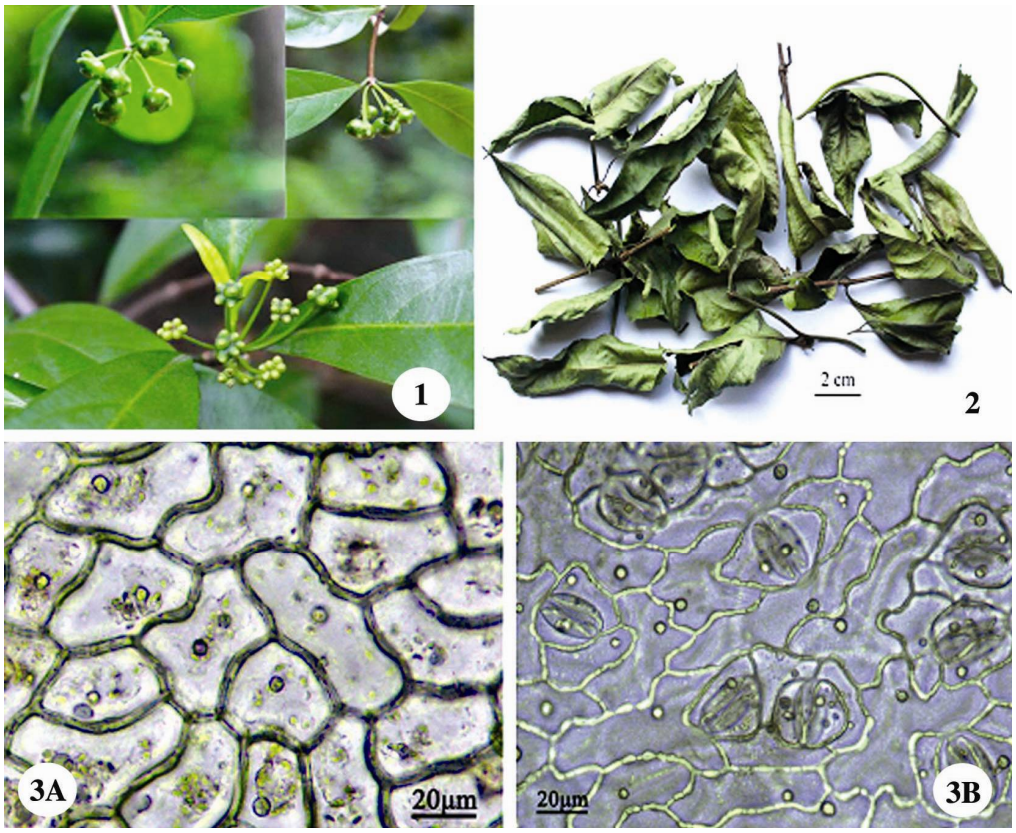
P-F is forward primer of *psbA-trnH* sequence. P-R is reverse primer of *psbA-trnH* sequence.
ITS-F is forward primer of ITS sequence. ITS-R is reverse primer of ITS sequence.

Results and Discussion

Morinda brevipes is a liane. The twigs are brown in color and slightly lignified. The blades are obovate oblong circle, obovate, oblanceolate, lanceolate in shaped, papery or subleathery, measuring 5 - 10 cm in length and 2 - 3 cm in width with characteristic of apex acuminate or acute, base cuneate and margin entire. Petiole ranges from 5 - 10 mm in length which are covered with

short coarse hairs (Fig. 1). Fruits are drupes which are formed by the development of many flowers with subglobose shape, measuring 10 - 12 mm in diameter. When drupes are matured, the color of fruits becomes orange or orange red. Drupes with 2 - 4 pyrene are nearly three prisms. Twigs are densely covered with short coarse or short fine hairs while old branches are glabrousness, slightly lignified and brown in color. The color of upper surface is grass yellow or brown black whereas underside is green, brown, or brownish red. It has a mild odor and a light taste (Fig. 2). The macroscopic characteristics of plants have particularity which can provide an intuitionistic basis for the identification of authenticity.

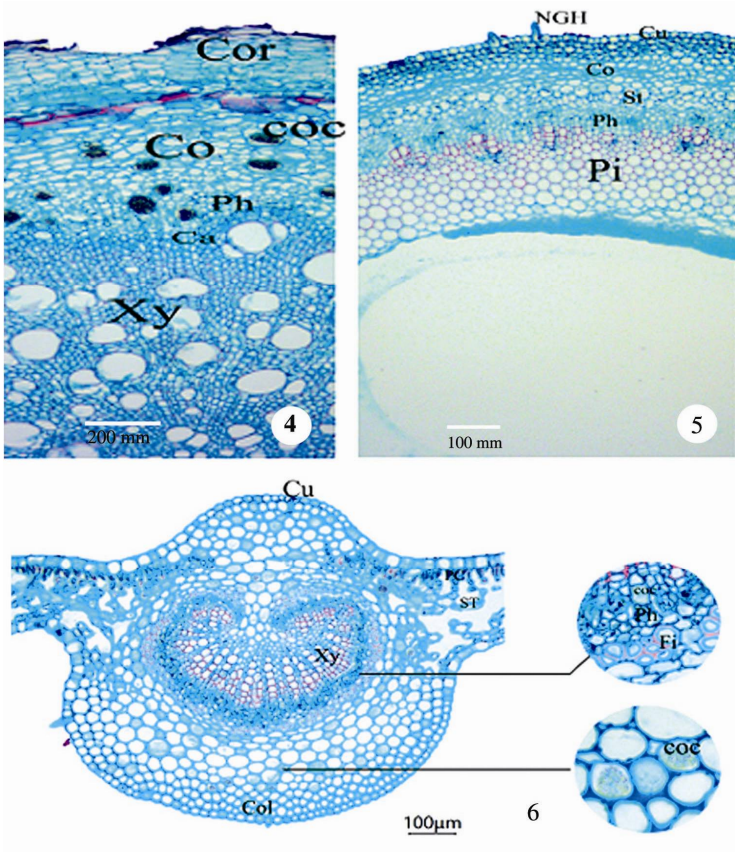
The upper epidermis cells are rectangular in shape and cuticle have clear texture (Fig. 3A). The lower epidermis cells have irregular form with many stoma of paracytic type surrounded by two or three subsidiaries (Fig. 3B).Transverse section of root is circular having the outline of epidermis, cortex and xylem. The epidermis is broken and obliterated. Cork layer is made up of many layers of applanate cells. Stone cells groups are arranged into a ring inside the cortex and calcium oxalate



Figs 1-3: 1. Fresh plant of *Morinda brevipes*. 2. Dried plant of *M. brevipes* and 3. Epidermis of leaf.

sandy crystal can be seen in parenchyma cell. The phloem shows narrow width. Cambium has ring shape. The root has broad xylem. Single vessel is scattered, or two to three vessels are aggregated (Fig. 4). Transverse section of stem is circular having the outline of epidermis, cortex, and xylem. Epidermis are made up of a list of similarly square cells of compact arrangement which has non-glandular hair outside. The cortex had slightly broad width and the parenchyma cells were

closely arranged. The stem showed the presence of narrow phloem. The cambium was irregular and wavy shape. The xylem shows a series of cells with broad width which formed a ring. The middle of the stem was hollow (Fig. 5). Epidermis of leaf were made up of a list of similarly square cells which had non-glandular hair outside. The palisade tissue was found to be differentiated from the spongy tissue. Palisade tissue was composed of 1 to 2 rows short cylindrical cells while spongy tissue cells were loosely arranged. The lower and above epidermis was one to series of collenchyma on the main vein. Xylem had a width of 5 to 8 rows of cells that is concave in shape and lignified. The blade showed narrow phloem which is located on the periphery of xylem. Calcium oxalate square crystal can be seen in cell. The lignified fibers were located on the periphery of phloem. Calcium oxalate sandy crystals are scattered in parenchymatous cells (Fig. 6).



Figs 4-6.: 4. Transverse section of root. Cor: Cork, COC: Calcium oxalate crystal, Co: Cortex, Ph: Phloem, Ca: Cambium and Xy: Xylem. 5. Transverse section of stem. NGH: Non-glandular hair, Cu: Cuticle, Co: Cortex, St: Stone cell, Ph: Phloem, Ca: Cambium and Pi: Pith. 6. Transverse section of blade. Cu: Cuticle; PC: Palisade tissue; ST: Spongy tissue; Xy: xylem; COC: Calcium oxalate crystal; Ph: Phloem; Fi: Fiber; NGH: Non-glandular hair and Col: Collenchyma.

Cork cells were rectangle in shape and yellowish-brown in color. Longer fibers were pale green. Most of the fibers were singly scattered. Fibers were 5 to 26 µm in diameter. Xylon was yellowish-brown and the diameter were 23 - 55 µm. Most of the xylon remain as bunch. Vessels were spiral having the diameter 16 - 45 µm. Pitted vessel was rarely seen with clear pit aperture.

Stone cells are inconsistent in size, singly scattered and square or rectangluar in shape. Stone cells are class-square and rectangle in shape with thicker walls and clear furrows. Tiny Calcium oxalate needle crystal was usually singly scattered . Calcium oxalate sand crystal occasionally exists in parenchyma cells which was granular (Fig. 7). Microscopic characteristics of this plant might serve as important distinguishing feature to recognize them from other plants of the same genus.

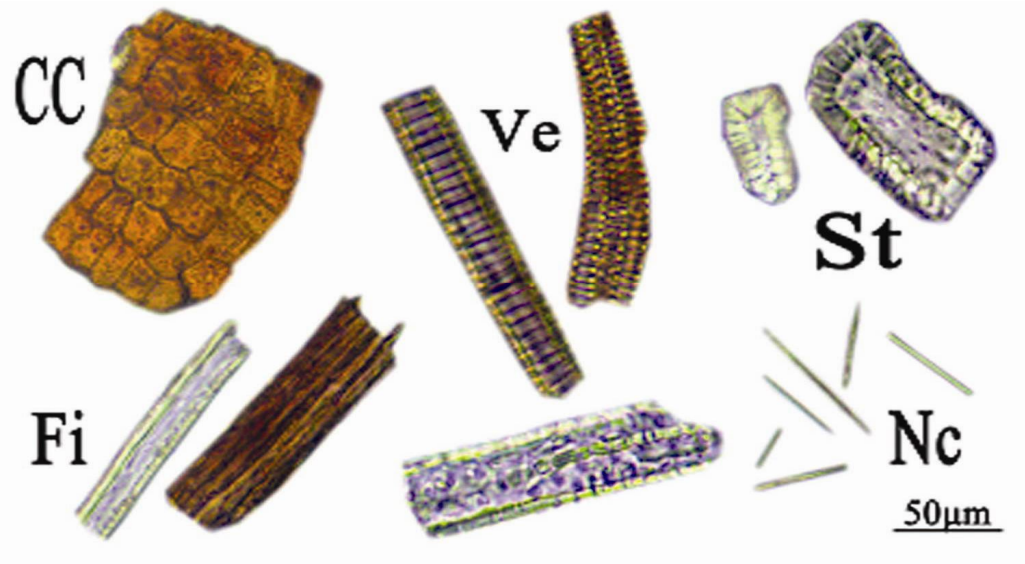


Fig. 7. Powder analysis. CC: Cork cell, Fi: Fiber, Ve: Vessel, St: Stone cell and NC: Needle crystal.

Table 2. Preliminary phytochemical screening of *Morinda brevipes*.

Phytochemicals	Test	Observations	Results
Sugar/glycosides	Molisch's test	Purplish-red ring appeared	+
Amino acid/ Polypeptide/ proteins	Ninhydrin test	Purplish-red or blue color observed	-
Saponins	Froth formation test	Lots of forth formed for 10 min	+
Tannins	$FeCl_3$ test	Dark green color appeared	+
Volatile oil and fats	Filter paper test	Oil spot appeared	+
	Hydrochloric acid - Mg test	Red forth	-
Flavonoids	Aluminum trichloride test	Yellow fluorescence	+
	Ammonia fumigation test	Yellow fluorescence	-
Anthraquinones	Lye test	Red forth	+
Phytosterols/ triterpenoids	Acetic anhydride - concentrated sulfuric acid test	No obvious color changing enhanced	-
	Iodine-potassium iodine test	Brown precipitate	+
	Silicotungstic acid test	Pale yellow or white precipitate	+
Alkaloids	Phosphomolybdic acid test	Pale yellow or white precipitate	+

+ indicates present and - absent.

Morinda brevipes were extracted with water and petroleum ether which were identified by characteristic reaction. The results showed that carbohydrates, saponins, tannins and volatile oils are present in this plant. Flavones and anthraquinones were found in ethanolic extract while acid-water soluble extract revealed the presence of alkaloids (Table 2). The Fig. 8 showed that the ethanol extracts of *M. brevipes* represented five distinct TLC spot on the silica gel plate. The TLC spot representing isolated compound with specific Rf values. The sample spots and the emodin reference substance are shown on the silica gel plates which have the same color and rf value (rf = 0.51). The result of thin layer chromatogram showed the presence of emodin in *M. brevipes*.

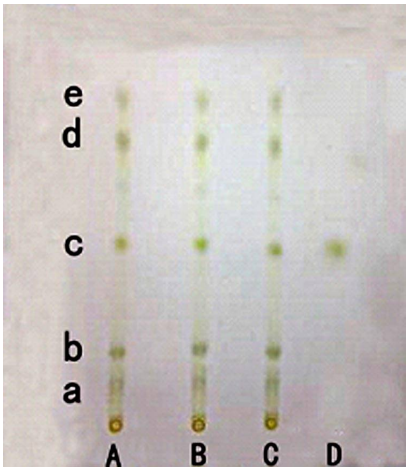


Fig. 8. TLC of *Morinda brevipes*: a,b,c,d,e representing isolated compound with specific rf values. A, B, C - ethanol extracts of *M. brevipes*, D - standard of emodin.

The ITS and *psb*A-*trn*H sequence of *M. brevipes* were extracted for the first time and submitted to NCBI to obtain the GenBank registration number which were MG996736 and MK409382, respectively. Their nucleotide sequence turned to be novel and did not have homology with any coding sequences of structural. The results of amplified sequences of ITS and *psbA- trnH* region for *M. brevipes* are presented in Table 3. It can provide a basis for the interspecific genetic study of medicinal plants.

Table 3. Amplified results of ITS and *psb*A-*trn*H region for *M. brevipes.*

Region	Base content (%)				Amplicon length (bp)	GenBank accession no.
	A	C	G	T		
ITS	15.3	30.9	32.8	21.1	697	MG996736
*psbA-trn*H	33.5	10.7	17.4	38.5	381	MK409382

The information obtained from overall traditional pharmacognostic identification and DNA barcoding studies of *M. brevipes* might play a significant role in authentication of the plant and standardizing herbal drugs.

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