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Pharmacognostic studies on three species of Spermacoce

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ABSTRACT

To provide a scientific basis for identification of *Spermacoce* species, including *Spermacoce remota* Lamarck, *Spermacoce exilis* (L. O. Williams) C. D. Adams, and *Spermacoce alata* Aubl., the current study was carried out for pharmacognostical parameters. Roots, stems, and leaves of plants were collected for pharmacognostical studies involving source identification, microscopic evaluation, character observation, phytochemical screening, ultraviolet spectrum and molecular pharmacognosy analysis. *S. remota*, *S. alata*, and *S. exilis* belong to the same genus, and the morphology of the plants was similar. Powder microscopy showed the presence of fibers, needle crystal bundles, spiral vessels, and near spherical pollen grains in all the plants. Phytochemical investigation demonstrated that *S. remota*, *S. alata*, and *S. exilis* all contained glycosides, proteins and volatile oils. In addition, the extracts of *S. alata* and *S. exilis* revealed the presence of flavonoids and tannins. In this study, the ITS sequence of *S. exilis* was found for the first time which had been submitted to NCBI to obtain the GenBank registration number. The results of neighbor-joining phylogenetic tree showed that *S. exilis*, *S. remota* and *S. alata* could be distinguished accurately. The study will be beneficial to the identification, resource conservation and quality control of *Spermacoce* species.

1. Introduction

The *Spermacoce* species in the family Rubiaceae are herbs or low shrubs, comprising approximately 275 species, widespread in tropical and subtropical regions around the world.[1,2] Leaves are opposite each petiole. The inflorescence is terminal or axillary, capitate or glomerulate, and many-flowered. Corolla is salverform or funnelform with white color. Fruits are capsular, which are ellipsoid to subglobose in shape. Some species in this genus can be used for the treatment of malaria, fever, headache, and respiratory infections.[3] Modern studies demonstrated that the main chemical compounds of the *Spermacoce* genus are flavonoids, alkaloids, phenols, and tri-terpenoids.[4,5]

Spermacoce alata Aubl. (Synonym: *Borreria alata* (Aubl.) DC., and *Spermacoce latifolia* Aubl.), is a potential medicinal plant locally known as "Sifanggucao" in China, belonging to the genus *Spermacoce* in the family Rubiaceae. It is native to South America, and is considered an exotic invasive in tropical and subtropical countries. In 1937, *S. alata* was introduced into Guangdong Province of China as military horse feed. Now, it has escaped into the wild. *S. alata* has been highlighted the use of its roots for the treatment of malaria. The ethanol extract of *S. alata* showed antibacterial activities against *Staphylococcus aureus*, *Bacillus cereus*, and *Bacillus subtilis*.[6] *S. exilis* is an underutilized medicinally important plant that has been confirmed to possess antibacterial, antioxidant and anticancer activities, containing various active components such as ursolic acid, stigmasterol and hexadecanoic acid.[7,8] These studies indicated that *S. alata* and *S. exilis* have vast medicinal value. It is noteworthy that as plants of the same genus, *S. remota* also has high research value. However, the information on pharmacognosy identification of these three plants is very scanty. In view of the importance of knowing about the genuineness of these three plants, herein we made a detailed investigation on the source, characters, microscopic, physicochemical parameters, and DNA barcodes. This will help to classification and authentication of these plants.

2. Materials and methods

2.1. Collection and identification of plant material

The fresh plants of *Spermacoce remota* Lamarck were collected in the Pharmaceutical Botanical Garden of Guangdong Pharmaceutical University (23.05°N, 113.41°E), *Spermacoce exilis* (L. O. Williams) C. D. Adams were collected in the South China Botanical Garden of Chinese

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Academy of Sciences (23.18°N, 113.36°E), and *Spermacoce alata* Aubl.- were collected in the Huolu Mountain Forest Park of Guangzhou (23.18°N, 113.39°E) in 2020, identified by Prof. Shengguo Ji, School of Traditional Chinese Medicine, Guangdong Pharmaceutical University.

2.2. Traditional pharmacognosy analysis

Morphological characteristics of plants were studied by visual inspection, and the microscopic characteristics were observed by microscope. The dried plants were made into coarse powder for UV analysis and phytochemical investigation.[9]

2.3. DNA extraction, amplification and sequencing

A proper amount of liquid nitrogen was added to the freeze fresh plants which were fully ground to powder.[10] Three samples of each plant were selected for repetitive experiments. The total DNA was extracted by DNA extraction kit (ZP309-2, Beijing Zhuangmeng International Biology Gene Technology Co., Ltd.). ITS-f (forward: 5′-TCCTCCGCTTATTGATATGC-3′) and ITS-r (reverse: 5′-GGAAGTAAAAGTCGTAACAAGG-3′) primers, were used for amplification and sequencing. The amplification profile was 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s with a final extension step at 72 °C for 5 min. The sequencing work was completed by Beijing Liuhe Huada Gene Technology Co., Ltd.

2.4. Data analyses

The base quality value greater than 20 was taken as the standard to proofread and splice the sequence. DNAMAN was used for multiple sequence alignment and error checking after splicing. Phylogenetic and molecular evolutionary analyses were carried out using MEGA software version 11.0. The neighbor joining (NJ) tree was constructed with 1000 bootstrap replications to analyze the phylogenetic relationship among medicinal plants of *Spermacoce*. Genbank accession numbers cited in this study are shown in Table 1.

3. Results

3.1. Source identification

3.1.1. S. remota

S. remota is a suberect herb with a height of 30–60 cm, pertaining to Rubiaceae. Leaf blades are puberulent to glabrescent, with narrowly elliptic or lanceolate in shape. Apex is acute while the base is acute to cuneate in shape with 2 or 3 pairs of secondary veins. Stipules are membranous, and covered with coarse hairs. The stem is quadrangular to cylindrical, with obvious ridges, glabrous or ciliolate on angles. The inflorescence is terminal and in uppermost leaf axils. Its corolla is funnelform with white color. The calyx limb is 4-lobed. Seeds are brownish-yellow in color and obtuse at both ends, somewhat shiny, transversely ruminate-rugose with irregular deep grooves (Fig. 1A).

Table 1
GenBank accession number information of ITs sequences.

Number	Species	GenBank accession no.
1	*Spermacoce erosa*	AM939537
2	*Spermacoce breviflora*	KF737019
3	*Spermacoce prostrata*	AM939541
4	*Spermacoce prostrata*	KF736996
5	*Spermacoce glabra*	KF737022
6	*Spermacoce confusa*	KF737020
7	*Spermacoce tenuior*	KF737023
8	*Spermacoce alata*	KF736994
9	*Spermacoce alata*	MH050295
10	*Spermacoce capitata*	KM215380
11	*Spermacoce capitata*	MF166822
12	*Spermacoce capitata*	AM939536
13	*Spermacoce ocymifolia*	AM939463
14	*Spermacoce remota*	AM939542
15	*Spermacoce remota*	FJ695467
16	*Spermacoce pusilla*	MH768318
17	*Spermacoce hispida*	AM939540
18	*Spermacoce flagelliformis*	AM939538
19	*Spermacoce ruelliae*	AM939543
20	*Spermacoce filituba*	AM939539
21	*Coffea lebruniana*	FR832863

3.1.2. S. exilis

S. exilis is a prostrate herb with a height of 15–60 cm, pertaining to Rubiaceae. Leaves are membranous nearly smooth, with ovate or oblong-elliptic in shape. Apex is acute to obtuse while the base is cuneate to obtuse in shape. Stipules are hirtellous with 5–10 bristles. The stem is quadrangular and covered with hairs. The inflorescence is terminal and in uppermost leaf axils, many-flowered, with white corolla. The calyx limb is 2-lobed. Seeds are brownish yellow, ellipsoid, obtuse at both ends, shiny, and surface apparently with numerous fine horizontal striations or ridges (Fig. 1B).

3.1.3. S. alata

S. alata is an erect to weak or clambering herb with a height of 20–60 cm, pertaining to Rubiaceae. Leaves are elliptic or ovate-oblong in shape, which are larger than other plants. Apex is acute or obtuse while the base is cuneate to obtuse in shape with 5 or 6 pairs of secondary veins. The stem is quadrangular and covered with hairs. The inflorescence is terminal and in uppermost leaf axils, measuring 5–12 mm in diameter. The calyx limb is 4-lobed. Corolla is white or light purple in color, with the funnelform puberulent outside. Seeds are brown in color, ellipsoid, measuring 2–4 mm long, obtuse at both ends and somewhat shiny with a longitudinal groove in the middle (Fig. 1C).

3.2. Medicinal material character identification

3.2.1. S. remota

Roots are solid with longitudinal wrinkles, less developed, while showing brownish-red or brownish-yellow in color. The fracture surface is not flat. Stems are green or brownish-red in color with a cylindrical shape, and easy to break up by hand. The upper surface of the leaf is green, and the bottom is light green. It has a faint smell and bitter taste (Fig. 1A).

3.2.2. S. exilis

The medicinal material is light and crisp, and the roots are yellowish with an uneven section. Stems are yellowish green or light green in color with a quadrangular shape, easy to break up by hand. Leaves are ovate or elliptic-oblong in shape, smooth and glabrous, measuring 0.5–1.5 cm long and about 0.5 cm wide. It has a faint smell and slightly bitter taste (Fig. 1B)

3.2.3. S. alata

The medicinal material is light. Roots are solid, and not easy to break. Stems are greenish to yellow-green in color with a quadrangular shape, easy to break up by hand. Leaves are papery, and the intact of which is flattened to be ovate. It has a slight odor and tastes bitter (Fig. 1C).

3.3. Microscopic characters

3.3.1. S. remota

The transverse section of stem is somewhat quadrangular, having the outline of epidermis, cortex, and xylem. The epidermis comprises one layer of Sub-elliptical or long elliptical cells arranged densely surrounded by cuticle. The cortex is made up of 6–10 layers of collenchymatous cells, with many calcium oxalate cluster crystals, needle crystal bundles and reddish-brown stuff, while the phloem is narrow with reddish-brown

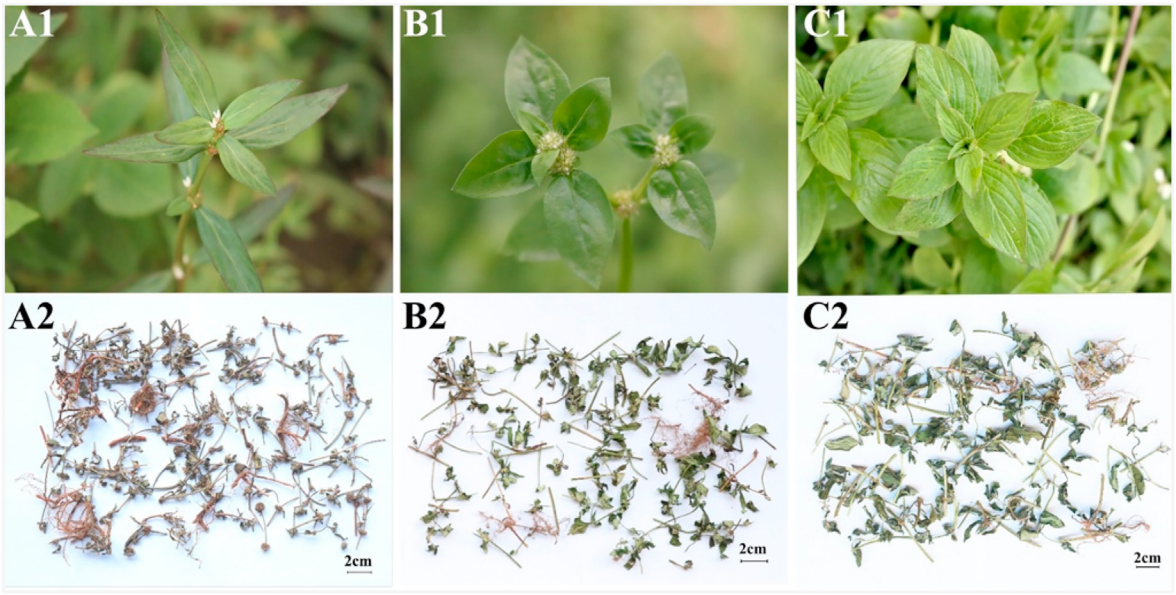

Fig. 1. The plants of (A) *S. remota*, (B) *S. exilis*, (C) *S. alata*.

stuff. Vessels are arranged radially in the xylem. At center, the pith is broad with reddish-brown stuff. Both the upper epidermis and lower epidermis of main vein consist of a layer of tidily and densely arranged cells. Beneath the epidermis of the main vein is a series of collenchyma cells. The palisade tissue consists of one or two layers of rectangular cells, while sponge tissue is loose (Fig. 2A). The upper epidermis cells are polygonal-shaped form. The lower epidermis cells are irregularly polygonal form with stomata, which are paracytic type, and the stomatal index is 24.4%–26.8%. The powder is greenish brown in color. Microscopic powder study reveals the presence of needle crystal bundles, pollen grains, fibers, and vessels. Many needle crystal bundles remain as bunches or scattered with a length of 30–45 μm. There are spiral vessels and pitted vessels. Fibers are slender in shape with thick wall. Pollen grains are near spherical with three germination holes and a diameter of 25–45 μm (Fig. 3).

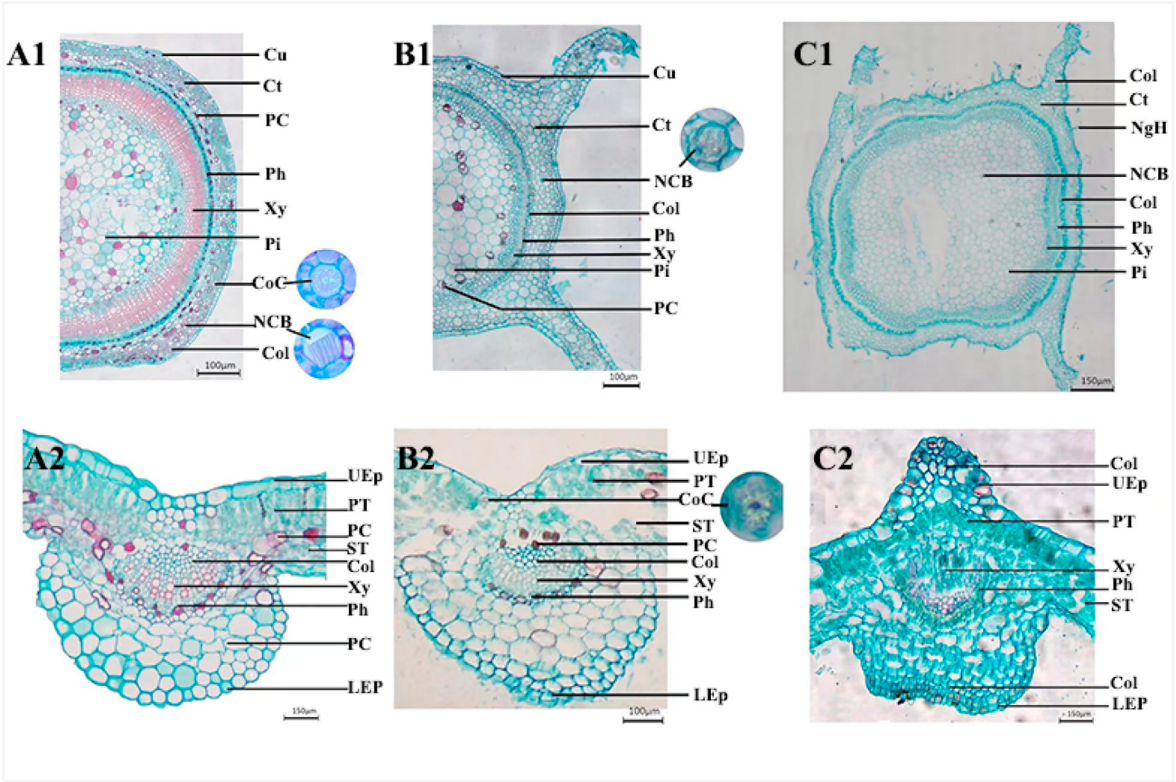

Fig. 2. The plants of (A) *S. remota*, (B) *S. exilis*, (C) *S. alata*. 1: Transverse section of stems, 2: Transverse section of leaves. Cu: cuticle, Ct: cortex, PC: parenchyma cell, Ph: phloem, Xy: xylem, Pi: pith, COC: calcium oxalate cluster crystals, NCB: Needle crystal bundles, Col: Collenchyma, NgH: non-glandular hair, UEp: upper epidermis, PT: palisade tissue, ST: spongy tissue, LEp: lower epidermis.

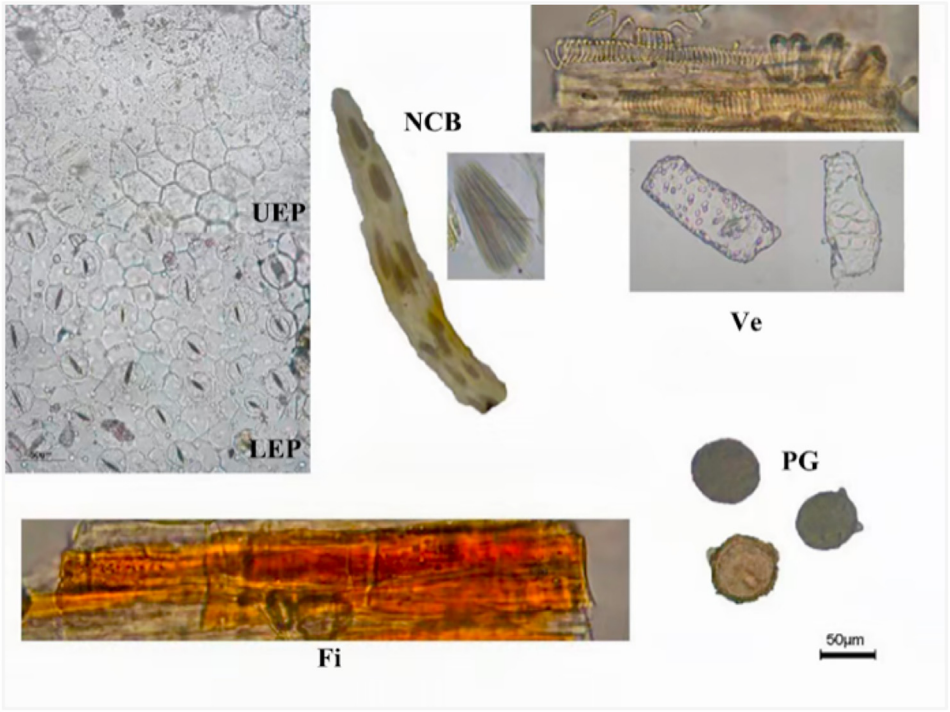

Fig. 3. Epidermis of leaf and powder microscopy of *S. remota*. UEp: upper epidermis, LEp: lower epidermis, PG: pollen grains, Ve: vessels, Fi: fibers.

3.3.2. *S. exilis*

The microscopic characteristics of *S. exilis* are similar to *S. remota*. However, the cortex of *S. exilis* is relatively thinner than *S. remota*. The Cortex is made up of 4–6 layers of collenchymatous cells. 2–4 vessels are arranged radially in the xylem, of which cell walls are lignified. Both the upper epidermis and lower epidermis of main vein consist of a layer of tidily and densely arranged cells, which have non-glandular hairs outside. Single vessel is scattered, or two to three vessels are aggregated (Fig. 2B). Both the upper and lower epidermis cells are irregular-shaped form. The lower epidermis cells have stomata, which are paracytic type surrounded by two subsidiaries, and the stomatal index is 27.1%–28.5%. The powder is green in color. Microscopic powder study reveals the presence of needle crystal bundles, pollen grains, fibers, vessels, and non-glandular hairs. Many needle crystal bundles remain as bunches or scattered with a length of 35.7–95.2 μm. There are a few non-glandular hairs, composed of 2–4 cells, measuring 120–320 μm long. Pollen grains are spherical in shape and light yellow in color, with a diameter of 16.2–22.9 μm (Fig. 4).

3.3.3. *S. alata*

Transverse section of stem is quadrangular. The epidermis is made up of some rectangular cells of compact arrangement which has non-glandular hairs outside. The cortex has needle crystal bundles, while the phloem is narrow. Vessels are arranged radially in the xylem, and the pith is broad. Both the upper epidermis and lower epidermis of main vein consist of a layer of tidily and densely arranged cells. Beneath the epidermis of the main vein several layers of collenchyma cells. Needle crystal bundles and calcium oxalate cluster crystals are scattered in parenchymatous cells (Fig. 2C). Both the upper and lower epidermis cells are irregular-shaped form. The lower epidermis cells have stomata, which are paracytic type surrounded by two subsidiaries, and the stomatal index is 21.4%–28.4%. The powder is grayish green. Microscopic powder study reveals the presence of needle crystal bundles, calcium oxalate cluster crystals, pollen grains, fibers, vessels, and non-glandular hairs. There are a great number of needle crystal bundles with a length of 29–78 μm, while calcium oxalate crystals are less. Most vessels are spiral-shaped. Fibers are flat and straight with noticeable pits. Pollen grains are near spherical with three germination holes and a diameter of 25–40 μm (Fig. 5).

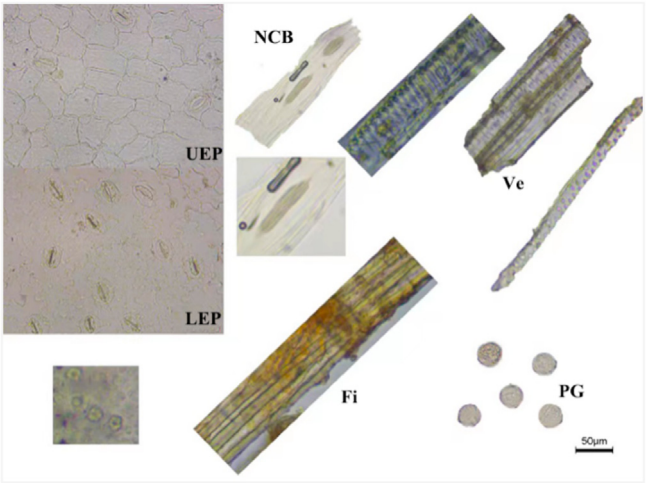

Fig. 4. Epidermis of leaf and powder microscopy of *S. exilis*. UEp: upper epidermis, LEp: lower epidermis, NCB: Needle crystal bundles, PG: pollen grains, Ve: vessels, COC: calcium oxalate cluster crystals, Fi: fibers.

3.4. *Phytochemical screening*

The results of phytochemical screening revealed that the three plants all contained glycosides, proteins and volatile oils. In addition, the extracts of *S. alata* and *S. exilis* revealed the presence of flavonoids and tannins (Table 2).

3.5. *UV absorption spectrum*

The UV absorption spectra of the three plants were shown in Fig. 6, Fig. 7, and Fig. 8. The 95% ethanol extract, ethyl acetate extract, and

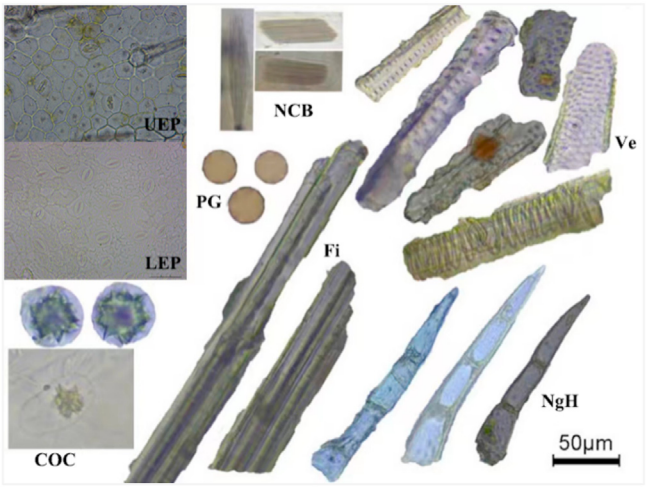

Fig. 5. Epidermis of leaf and powder microscopy of *S. alata*. UEp: upper epidermis, LEp: lower epidermis, NCB: Needle crystal bundles, PG: pollen grains, Ve: vessels, COC: calcium oxalate cluster crystals, NgH: non-glandular hair.

chloroform extract of *S. remota* possessed strong absorption values near (668 nm, 414 nm, and 242 nm). *S. exilis* and *S. alata* exhibited strong absorption values near (667 nm, 415 nm, 320 nm, and 210 nm) and (666 nm, 415 nm, 409 nm, and 207 nm), respectively.

3.6. ITS region sequence analysis

The sequence length of the three plants was close, about 660 bp, while the contents of G, C, A and T were about 27%, 28%, 23% and 21%, respectively, with high similarity, which could be used as a reference for DNA barcoding identification. In this study, the ITS sequence of *S. exilis* was obtained for the first time (GenBank registration number: OR841337). Compared with the sequence of the same plant uploaded on NCBI, it was found that the similarity between *S. remota* (extracted in this experiment) and *S. remota* (GenBank registration number: LN898443) was 100%, and the similarity between *S. alata* (extracted in this experiment) and *S. alata* (GenBank registration number: MH050295) was 100%. There was no genetic difference (Table 3).

3.7. Phylogenetic tree

Coffea lebruniana, belonging to the genus *Coffea* in the family

Table 2
Preliminary phytochemical screening.

Phytochemicals	Test	*S. remota*	*S. exilis*	*S. alata*
Sugar/glycosides	Molish reaction	Purplish-red ring appeared	Purplish-red ring appeared	Purplish-red ring appeared
Amino acid/Polypeptide/ proteins	Ninhydrin test	Purplish-red or blue color observed	Purplish-red or blue color observed	Purplish-red or blue color observed
Saponins	Froth formation test	No phenomena	No phenomena	No phenomena
Tannins	$FeCl_3$ test	No phenomena	Dark green color appeared	Dark green color appeared
Volatile oil and fats	Filter paper test	Oil spot appeared	Oil spot appeared	Oil spot appeared
Flavonoids	Aluminum trichloride test	No yellow or sky blue fluorescence observed	Yellow fluorescence	Yellow fluorescence
Alkaloids	Silicotungstic acid test	No phenomena	No phenomena	No phenomena
	Phosphomolybdic acid test	No phenomena	No phenomena	No phenomena
	Iodine-potassium iodine test	No phenomena	No phenomena	No phenomena

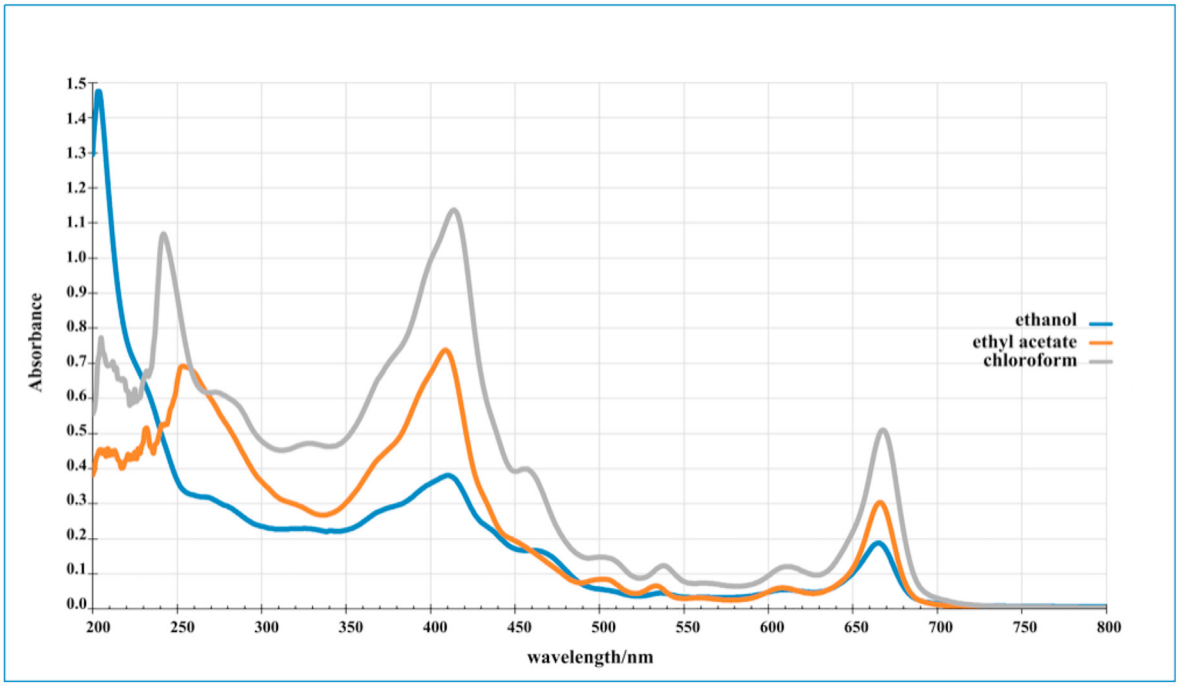

Fig. 6. UV absorption spectrum of *S. remota*.

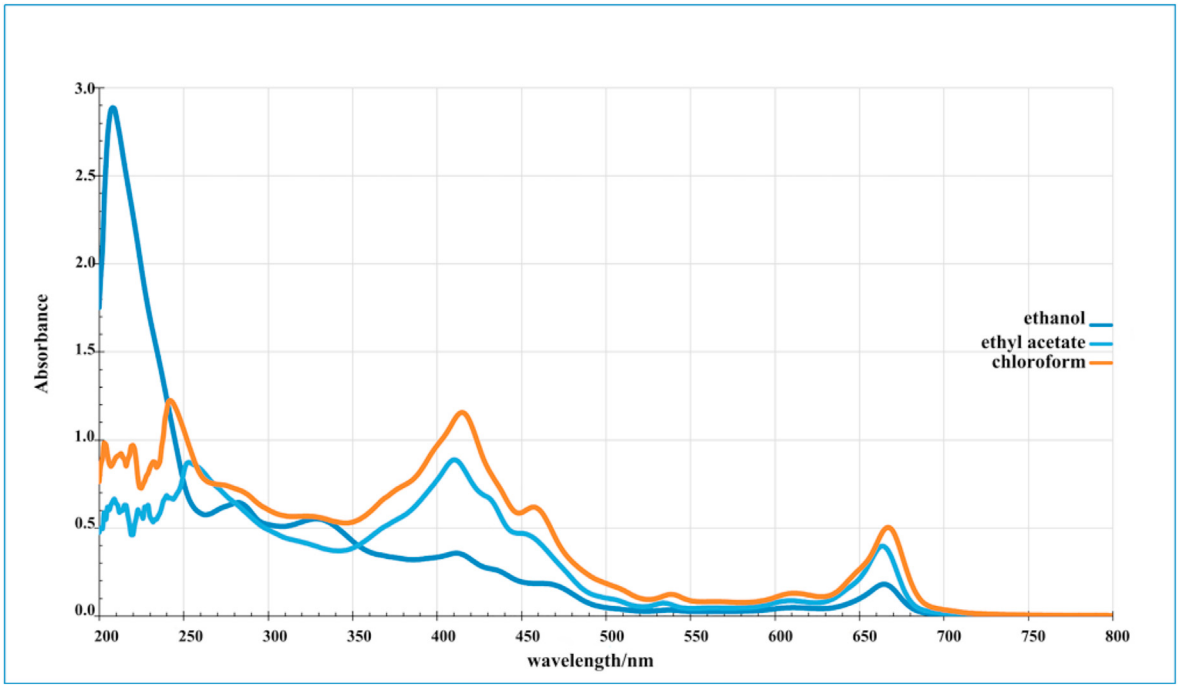

Fig. 7. UV absorption spectrum of *S. exilis*.

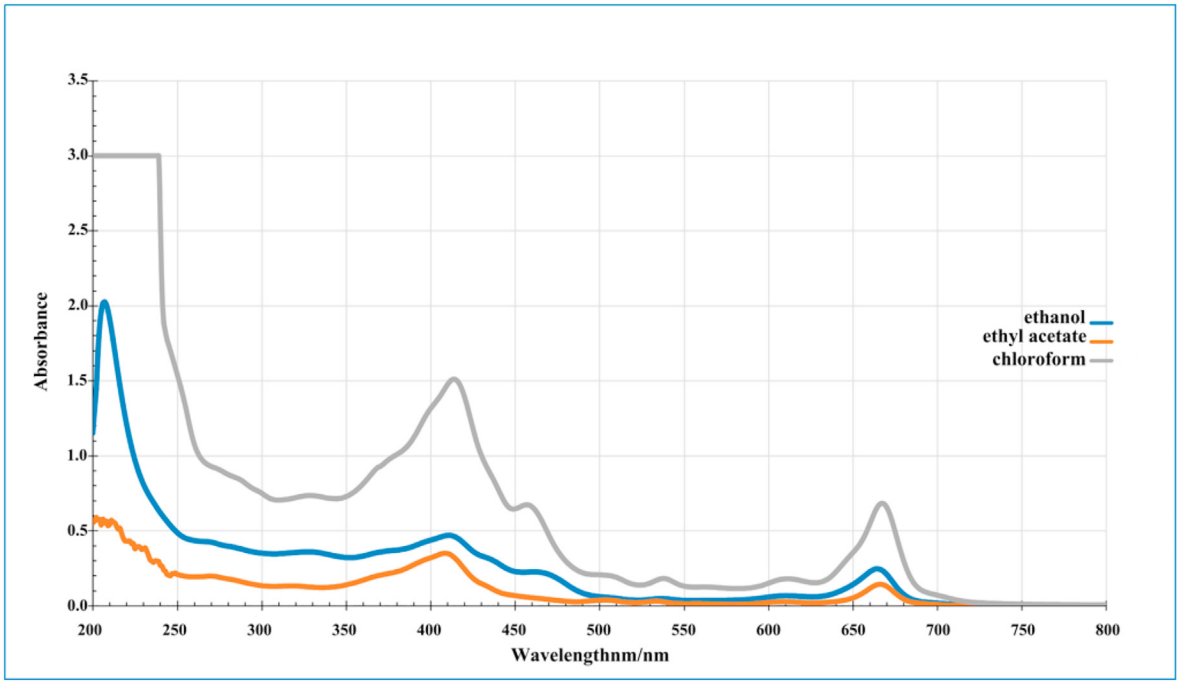

Fig. 8. UV absorption spectrum of *S. alata*.

Table 3
Amplified results of ITS region.

Region	Base content (%)				Amplicon length (bp)
	A	C	G	T	
S. remota	24.1	28.1	27.4	20.5	665
S. exilis	20.3	28.4	29.7	21.6	640
S. alata	23.2	28.8	27.3	20.7	671

Rubiaceae, was closely related to the three *Spermacoce* species studied in this experiment, which was beneficial to infer the evolution history of *Spermacoce* plants accurately, so it was selected as outgroup taxon to construct the NJ phylogenetic tree. The results showed that plants were clustered into three branches with bootstrap values of 71%, 85% and 59%, in which *S. remota* and *Spermacoce pusilla* (MH768318) were clustered into a small branch, while *Spermacoce erosa* (AM939537), *Spermacoce tenuior* (KF737023), *Spermacoce confusa* (KF737020) and *S. alata* were clustered into a branch with a bootstrap value of 71%, indicating

that *S. remota* was closely related to *Spermacoce pusilla* (MH768318), while *S. alata* was closer to *Spermacoce erosa* (AM939537), *Spermacoce tenuior* (KF737023), and *Spermacoce confusa* (KF737020) (Fig. 9).

4. Discussion

Medicinal plants are characterized by low side effects, and have been widely used in the prevention and treatment of various diseases.[11–13] High-quality medicinal materials exhibit better curative effects in the clinic, while counterfeit medicines can sharply reduce the efficacy or even cause poisoning.[14] Hence, it is critical to identify the varieties and control the quality of herbal medicines. However, in the modern medical system, due to the lack of pharmacognosy research, there has not been sufficient evidence for the standardization of *S. remota*, *S. alata*, and *S. exilis* in China. Considering these facts, this study attempts to find out the pharmacognostic characteristics of these plants, to identify and control the quality of plant materials. Source identification is a fundamental step for authentication of such plants, and plays an imperative role. *S. remota*, *S. exilis*, and *S. alata* all belong to the genus *Spermacoce*, family Rubiaceae. Morphological differences of the three plants are shown as follows: *S. remota* calyx limb is 4-lobed, the stem is quadrangular to cylindrical, with obvious ridges, glabrous or ciliolate on angles, and leaves are narrowly elliptic or lanceolate in shape; *S. alata* calyx limb is 4-lobed, the stem is quadrangular covered with hairs, and leaves are elliptic or ovate-oblong in shape, which are larger than other plants; *S. exilis* calyx limb is 2-lobed, the stem is quadrangular covered with hairs, and leaves are ovate or oblong-elliptic in shape, which are critical diagnostic characters. Microscopic studies, whose main features are fibers, ducts and non-glandular hairs, are accurate, reliable, rapid and pollution-free in determining the identity of the source materials. Preliminary phytochemical analysis of *Spermacoce* species showed that all contain glycosides, proteins and volatile oils. Among them, *S. exilis* extracts showed the presence of flavonoids and tannins as well, which is consistent with the results reported in previous literature.[15] Plant identification is often the comprehensive application and mutual verification of multiple methods.[16–18]

Traditional identification methods such as source identification, character identification and microscopic identification of plant materials have some limitations.[19–21] DNA barcoding technology is not affected by individual morphological characteristics, and the method is easily unified and standardized, which is an effective supplement to traditional identification methods.[22] At present, there is no literature on the utilization of ITS sequences for the identification of *S. remota*, *S. exilis*, and *S. alata*. In this study, the total DNA of three plants was extracted successfully, and the ITS sequence of *S. exilis* was obtained for the first time. After comparing the ITS sequences of *S. remota* and *S. alata* with the sequences of the same plant submitted to the NCBI database, it was found the similarity was 100%, indicating that the ITS sequence identification has high stability. The NJ phylogenetic tree based on the ITS sequence can effectively identify *S. remota* and its closely related species. In general, the information generated in this work may contribute to the further development of *Spermacoce* species, and it can be used as reference information to provide technical guarantee and scientific basis for rapid and accurate identification of plant materials.

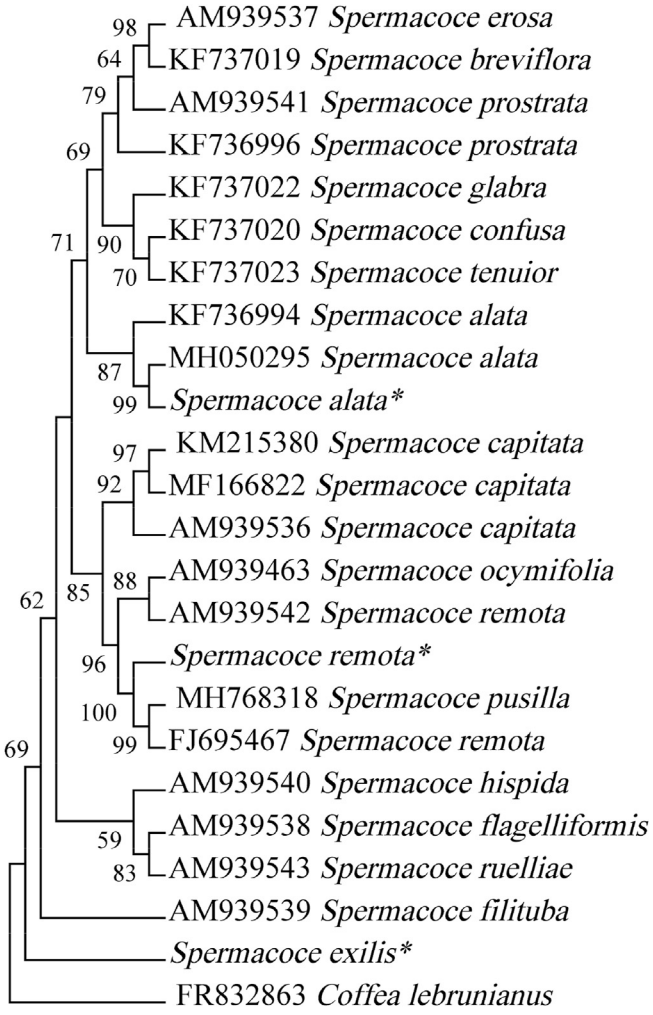

Fig. 9. Phylogenetic tree based on ITS sequences.

5. Conclusion

In the present study, evaluation of source, medicinal material character, microscopy, physicochemical parameters and DNA barcoding were carried out, and this could be helpful in authentication of *Spermacoce* species. Furthermore, the Phylogenetic tree of *Spermacoce* species was researched, which provided a reference for germplasm conservation and quality control. Above all, this study will help identify, standardize, and detect adulteration in plants.

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CRediT authorship contribution statement

Shan Fan: Writing – review & editing, Investigation, Data curation. **Wenfeng Weng:** Investigation, Data curation. **Shengguo Ji:** Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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