Pharmacognosy research and identification of *Euphorbia prostrata* Ait.

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Jia Yan wrote and revised the manuscript; Wen-Feng Weng contributed to drawing the diagram; Sheng-Guo Ji reviewed the manuscript and provided financial support. All authors read and approved the final version of the submitted manuscript.

Competing interests
The authors declare no conflicts of interest.

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Abbreviations
ITS, Internal Transcribed Spacer; PCR, Polymerase Chain Reaction; K2P, Kimura 2-Parameter; Mega, Molecular Evolutionary Genetics Analysis.

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Abstract
**Background:** *Euphorbia prostrata* Ait. is an annual herb widely distributed in the southern region of China with great medical values on Anti-inflammation, insect repellent, treatment of diarrhea. Despite its extensive uses as a traditional Chinese medicine, no systematic research on the identification of *E. prostrata* has been reported. **Methods:** The study aimed to establish an accurate identification system for *E. prostrata* through traditional pharmacognostical methods, including botanical origin, morphological characters, medicinal material characters, microscopic characters, physicochemical parameters determination, phytochemical screening, and DNA barcoding analysis. **Results:** Physicochemical results show that this plant likely contains flavonoids, anthraquinones, and other substances. The ITS loci of the nuclear genome and psbA-trnH loci of the chloroplast genome were selected and evaluated, which were the most variable loci. **Conclusion:** The findings of this study are expected to contribute to the development of species identification, as well as provide references for authenticity identification, genetic relationship analysis, and further utilization of *E. prostrata*.

**Keywords:** *Euphorbia prostrata* Ait.; microscopic identification; physico-chemical identification; ITS sequence; psbA-trnH sequence
Background

*E. prostrata* widely distribute in the southern region of China, Its morphological characteristics extremely similar with *Euphorbia serpens* H.B.K., *Euphorbia thymifolia* L. and *Euphorbia maculata* L. [1–3]. Not noly the difference between with them is whether there is tomentose on the stem and fruit but also the entire plant of creeping *E. serpens* is almost reddish or pink, with obvious glandular appendages; *E. thymifolia* has very fibrous roots; and *E. maculata* has a purple stain in the center of the leaf surface when it is fresh [4].

Therefore, this study determined the Latin scientific fective identification system of *E. prostrata* AIT. [5]. Its gene sequence for the nuclear genome as well as the psbA-trnH intergenic region for the chloroplast genome were also included. This information served as a reference for the genetic relationship, authenticity identification, and future development and use of *Euphorbia prostrata* AIT.

Methods

Collection and identification of plant material

As shown in Supplementary Table S1, all the fresh materials, including the roots, stems, and leaves of the plants were collected from the campus of Guangdong Pharmaceutical University and the South China Botanical Garden in Guangzhou, China. They were identified as *Euphorbia prostrata* Ait. (23.05'N, 113.41'E), *Euphorbia serpens* H.B.K. (23.05'N, 113.41'E), *Euphorbia thymifolia* L.(23.05'N, 113.41'E), and *Euphorbia maculata* L. (23.18'N, 113.36'E) by Professor Shengguo Ji. The plant specimens were preserved in the herbarium of the College of Traditional Chinese Medicine, Guangdong Pharmaceutical University. Observe the morphology of collected medicinal plants, take pictures, and describe and record the whole plant morphology, roots, stems, leaves, flowers, fruits and other organs in detail. By checking the literature, key and specimens, the species source and Latin name are determined. The general texture, color, and appearance of the medicinal materials, as well as the regional traits and cross-sectional morphology of roots, stems, and leaves, are observed, described in detail, and recorded after the medicinal plants have been collected and dried in the shade. Pictures of the medicinal materials are also taken and saved.

Preparation of sample

Source identification. Take fresh plant roots, stems, and leaves, clean them, and let them dry in the air. Then, choose the parts with complete root surfaces, 3–5 cm away from the roots or stems, and no or few root hairs. Then, choose the parts with complete stem surfaces, but not too close to nodes with the whole stem. Finally, choose the parts with complete leaf surfaces and mature development. Include chopping the main vein and both of the blades into small pieces that are 1 cm broad and 2–3 cm long, soaking them in FAA fix liquid for 24 hours, and sending them to Xinxing Heli Teaching Equipment Co., Ltd. for making permanent slices. The sections were observed under a biological microscope. (Nikon biological microscope, ECLIPSE E100, Beijing Century Kexin Scientific Instrument Co., Ltd.).

Leaf epidermis. Take more than ten plants of the same species, each plant has more than three fresh, complete and representative leaves, pick the upper and lower epidermis of the leaves with dissecting needles, and mount slices to observe the characteristics of epidermis, glandular scales, glandular hairs, non-glandular hairs and epidermal cells, such as shapes and stomatal types. And calculate that stomatal index by a formula.

\[ \text{Stomatal index} = \frac{\text{Number of pores per unit area}}{\text{Number of pores per unit area} \times \text{Number of epidermal cells in the same area} \times 1000} \]

Powder. Dry fresh plants in the shade, crush them with a pulverizer (Universal high-speed crusher, XPB-400, Huanan Zhongcheng Pharmaceutical Machinery Factory) and sieve them with a 100-mesh sieve. Take a proper amount of powder, and make tablets by common thin gelatin tablet method and chlorhydate permeation method.

The characteristics of milk dust, resin duct fragments, oil cells, glandular hairs, non-glandular hairs, fibers, stone cells, calcium oxalate cluster crystals, calcium oxalate needle crystal bundles, ducts and starch grains were observed and recorded under microscope.

Physical and chemical identification

Phytochemical tests.

General chemical composition identification of the powder by methods such as water extract, the molish reaction, the ninhydrin reaction, the ferric chloride reaction the foam reaction the filter paper test, the filter paper test, the AICI, reaction, the NaOH-HCl reaction, the Aetetic anhydride-concentrated sulfuric acid reaction, the Alkaline picric acid reaction, acid extrac, the Potassium iodide-iodine reaction, the Phosphomolybdic acid reaction, the Silico-tungastic acid reaction, the Modified bisbuth potassium iodide reaction.

Powder phenomenon. Take a little medicinal powder, and add it to seven kinds of solvents: glacial acetic acid, dilute sulfuric acid, dilute hydrochloric acid, dilute nitric acid, 9% ferric chloride, dilute sodium hydroxide, and dilute potassium hydroxide, respectively. Observe the phenomenon of powder (sinking/floatting) and the color change of the solvent, and record it.

Fluorescence phenomenon. Take 0.2 g crude powder of medicinal materials, add 10 mL of 75% ethanol, ethyl acetate, acetone, methanol, chloroform, carbon tetrachloride, distilled water and petroleum ether respectively, perform ultrasonic treatment for 1 h, take filtrates, place them in test tubes, observe the fluorescence color under sunlight and ultraviolet lamp at 254 nm and 365 nm respectively, and record.

UV-based spectroscopic analysis. Add 30 mL of 95% ethanol, ethyl acetate, and chloroform to 1 g of crude powder, ultrasonic for 30 min, filter, and put 0.4 mL of filtrate in a 10 mL volumetric flask to constant volume, which is the test solution. The test solutions were placed in Shi Yang cuvettes, and 95% ethanol, ethyl acetate, and chloroform were used as blank controls, which were scanned and measured by an ultraviolet spectrophotometer, and the spectra were measured in the wavelength range of 200-800 nm. Scanning parameters are set as follows: 200-800 nm wavelength range; Medium scanning speed; 1 nm scanning interval.

General inspection items.

The second method of Moisture Determination Method (Drying Method), specified in General Principles 0832, 2020 edition of the China Pharmacopoeia, was used to determine the moisture content of medicinal materials except chicken bone incense [6]. The water content of Jixiangxiang was determined by the toluene method, the fourth method specified in General Principles 0832, 2020 edition of the China Pharmacopoeia.

Determination of total ash and acid-insoluble ash: The ash content of medicinal materials was determined by the "total ash determination method" specified in General Principles 2302 of China Pharmacopoeia, 2020 [7].

Molecular marker identification

Sample processing. Select fresh plant leaves, wash the stains on the tissue surface with 75% ethanol, and absorb the liquid on the surface. Cut it into about 1 cm² size with scissors, put it in a freezing tube, store it in liquid nitrogen for later use, and grind it into fine powder with liquid nitrogen before extracting total DNA.

DNA extraction. Use the plant genome DNA extraction kit, and the specific operation is carried out according to the kit instructions.

Primer information. The ITS sequence of plant nuclear genome and the intergenic sequence of chloroplast psbA-trnH were amplified (PCR amplifiers, T100TM, Guangzhou Feidi Biotechnology Co., Ltd.) and sequenced by universal primers, and the primer sequences are as follows in Supplementary Table S2.

PCR amplification. Use the 2× Utaq PCR MasterMix kit, and the specific operation is carried out according to the kit instructions.

PCR reaction system. 1.0 μL upstream primer, 1.0 μL downstream primer, 1 μg total DNA extract, 2× master mix: 10.0 μL, make up to 25 μL with ddH₂O. PCR amplification conditions: pre-denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 min, annealing at 55 °C for 30 min, extension at 72 °C for 1 min, and extension at 72 °C for 5 min.

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after 30 cycles.

Agarose gel electrophoresis. After preparing 1× TAE buffer and 1% agarose gel, take 5 μL of PCR products, and electrophoresis them in an electrophoresis apparatus (125 V constant voltage, BG-power 300, Beijing Bajing Biotechnology Co., Ltd.) for 30 min. Using gel imaging analysis system (TOCAN240, Shanghai Lincheng Biotechnology Co., Ltd.), if the bands are clear and bright, send them to Guangzhou Branch of Beijing Liuhe Huada Gene Technology Co., Ltd. for sequencing.

Data analysis. Sequence comparison: Big Gene Company's gene proofreading and splicing software performs quality separation and proofreading and splicing, and proofreads and splices the sequencing sequences that meet the standard with the base quality value (QV) greater than 20 as the standard, removing primer regions and low-quality sequences. Use DNAMAN to perform multiple sequence alignment on spliced sequences, check for errors, and derive sequence alignment map. K2P (Kimura 2-Parameter, K2P) between sequences was calculated using software Mega 7.0 (Molecular Evolutionary Genetics Analysis), genetic distance was analyzed, and sequence alignment matrix was derived.

Results

Origin identification

The stem of this plant branches from the base and creeps around. The whole plant is reddish or red, and the stem is densely covered with white hairs. Leaves opposite, asymmetrical, slightly tapered, green, glabrous on both sides, leaf margin nearly entire or more than middle with irregular serrulate, apex with white hair; Stipules connate, Yangtze River Delta-shaped, split at the top, with white hair, easy to fall off. The fruit is triangular, its edges are covered with white hairs, and other parts are glabrous; Glands 4, purplish red, with pale pink appendages; Seeds are quadrangular, with 6–7 transverse grooves, pale white and brownish yellow.

Textual research on materia medica: Euphorbia prostrata was first published in Fujian Pharmaceutical Records: "light and cool," "clearing heat and cooling blood, detoxification and detumesence." Dysentery, enteritis, diphtheria, pharyngolaryngitis, chyluria, scanty milk, infantile malnutrition, bloody stool, bloody urine, bleeding gums, herpes zoster, dermatitis, eczema, carbuncles, and furuncles" (Figure 1).

Macroscopical identification

The Chinese medicinal material body is light and crisp. The root is slender, straight-rooted, occasionally branched, with a few fibrous roots or none, with thin longitudinal wrinkles on the surface, often bent, easily broken, uneven in section, and yellowish white; The stem is slender, smooth, with fine longitudinal stripes, white hair, a lavender or light green surface, easy to break, with an uneven section, light green, and occasionally a hollow center; The stem is smooth and has fine longitudinal stripes as well as white hairs. The leaves are curled, easy to fall off, easy to break, green on the leaf surface, pale green on the back of the leaves, hairless on both sides, occasionally white on the leaf margin, flat, oval or obovate, asymmetric at the base of the leaves, with irregular serrations all over or above the middle; The fruit is triangular, nearly spherical, yellow-green, with white hairs on the edge of the fruit; the seeds are quadrangular, brown or yellowish brown, with 6–7 transverse grooves. The smell is pale and the taste is slightly bitter (Figure 2).
Microscopical identification

The transverse section of the root is round-like. The cork cells are oblong or oblong, with 4–6 rows closely arranged tangentially, and the outermost cells are mostly broken. It is composed of rectangular, oval, or square-like parenchyma cells in the cortex. The cells are relatively flat and arranged in 4–8 tangential rows. The phloem cells are small, round, and arranged in 4–6 rows tangentially. The xylem is broad, accounting for most of the cross section, strongly lignified, with obvious rays and radial extension, with single or 2–7 ducts arranged in radial aggregation (Figure 3).

The transverse section of the stem is round-like. The epidermis is composed of a layer of oblong-like or oblong-like cells arranged tangentially, closely arranged, covered by cuticle, and with a small number of non-glandular hairs. The cortex is composed of 5–7 layers of oblong-like or oval-like thin-walled cells, with large cells. There are milk ducts scattered around the vascular bundles. The perithecal fiber bundles are arranged intermittently in a ring shape; the phloem is narrow; and 2–4 xylem vessels are arranged radially. The pith is sometimes broken and hollow (Figure 4).

Leaf cross-section: The upper and lower epidermis are composed of a row of cells, arranged in order; the upper epidermis cells are slightly papillar; the lower epidermis cells are round or oval; and the outer cuticle. The palisade tissue is composed of 1–2 rows of short cylindrical parenchyma cells, which pass through the main vein. Sponge tissue is narrow, and round parenchyma cells are loosely arranged with large gaps. Midvein is slightly depressed below. There are about 20 vascular bundles arranged in parallel on the leaf surface. The main vein vascular bundle has a large vascular bundle sheath and the phloem cells are small. One or two rows of phloem cells are arranged in a groove shape. There are milk ducts scattered around the vascular bundle, and the cells around the ends of veinlets are large (Figure 5).

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Leaf blade: the upper epidermal cells are irregular, the vertical wall is curved, the stomatal type is inequality or indefinite, and the stomatal index is 15.8%-17.4%. The lower epidermis cells are irregular, the vertical wall is curved, the stomata are indefinite, and the stomatal index is 16.0%-19.4%.

The powder microplate was green-brown. 1. Pollen grains are round or quasi-round, yellow, with 3 germinating holes, unsmooth surface, containing granular substances, with a diameter of 15.1–27.3 μm; 2. The epidermal cells of seed coat are golden yellow and stony; 3. Milk duct is often broken and contains particles; 4. The diameter of the threaded catheter is 15–25 μm; 5. There are granular protrusions on the surface of non-glandular hairs, which are 60–120 μm long; 6. Fiber bundles exist in bundles, with slightly thick walls and small cavities; 7. There are many cells around the end of veinlets, which are arranged in a round shape radially (Figure 6).

Physico-chemical identification
The results of preliminary physical and chemical identification tests are as follows: general chemical composition identification (Supplementary Table S2), powder phenomenon (Supplementary Table S3), and fluorescence phenomenon (Supplementary Table S4).

The 95% ethanol, ethyl acetate, and chloroform extracts of this product were examined by an ultraviolet spectrometer, and the results are shown in Figure 7. The results of the ultraviolet absorption spectrum showed that its 95% ethanol extract had two large absorption peaks at 413 nm and 665 nm. Ethyl acetate extract has two large absorption peaks at 410 and 666 nm. The chloroform extract has three big absorption peaks at 243, 415, and 243 nm.

Inspection: The water content, total ash content and acid-insoluble ash content of China Pharmacopoeia (2020 edition) are 6.13%, 16.39% and 7.62%, respectively.

Molecular marker identification
PCR amplification analysis. In this study, Euphorbia prostrata, Euphorbia stolonifera, Euphorbia lanceolata, and Parthenocissus tricuspidata, which belong to the Euphorbiaceae, were selected, and the whole sequence of ITS nuclear genome and psbA-trnH sequence of the chloroplast intergenic region were extracted, and DNA barcode identification technology was used as a supplement to traditional Chinese medicine identification technology. To provide reference and data support for the later development and utilization of Parthenocissus tricuspidata, the evaluation and formulation of quality standards, and the establishment of a DNA barcode system for Chinese medicinal materials.

Analysis of the PCR amplification results. In this experiment, total DNA was extracted from Euphorbia prostrata, Euphorbia stolonifera, Herba Hedyotidis Diffusa, and Parthenocissus tricuspidata. Three plants from each plant were selected for repeated experiments. Two gene fragments, ITS2 and psbA-trnH, were amplified by PCR. The PCR products were detected by 0.1% agarose gel electrophoresis. The results showed that the bands of ITS sequence and psbA-trnH were between 500–750 bp and 500–250 bp. The sequencing results showed that some samples were directly sequenced, and some samples were obtained by gel cutting, purification, and sequencing, and high-quality sequencing peaks were obtained.

ITS sequence analysis. In this study, the ITS sequences of total DNA sequences of Euphorbia prostrata, Euphorbia stolonifera, Herba Hedyotidis Diffusa, and Parthenocissus tricuspidata were amplified and sequenced, and the ITS sequences of some plants were obtained. After comparing and analyzing the amplified sequences with CLUSTAL software, the ITS sequence lengths of these plants were determined (Supplementary Table S5).

The results showed that the total bases of ITS sequences of plants in the same family and genus were similar. The above ITS sequence was compared with the database in the gene bank (Supplementary Table S6).

It can be seen from the results that the ITS sequences of Euphorbia prostrata and Herba Centipeda are consistent with the existing sequences in the gene bank. Among them, the ITS sequence of Euphorbia stolonifera is 727 bp in length, which is 96.83% similar to AY971200.1 and has 23 mutation sites. Sequence variation may be caused by factors such as plant growth environment (temperature, ultraviolet, etc.) and chemical drugs.

psbA-trnH sequence analysis. The sequence of the psbA-trnH intergenic region of the sample obtained in this study was analyzed by CLUSTAL software, and the sequence of the psbA-trnH intergenic region of experimental plants was successfully obtained (Supplementary Table S7).

In this study, the results of the psbA-trnH sequence obtained were compared with those of the same species uploaded to the NCBI database by DNAMAN software. It was found that the regional differences of Euphorbia prostrata did not produce gene differences, and the similarity was 100% (Supplementary Table S8).

The psbA-trnH sequence of Euphorbia stolonifera is 616 bp in length, which is 3 bp less than KT072879.1 and 96.54% similar to KT072879.1. There are 7 mutation sites between them. The full length of psbA-trnH sequence of Parthenocissus tricuspidata is 691 bp, which is 1 bp more than that of LC191852.1, and the similarity is 99.71% compared with LC191852.1. There are two mutation sites between them. In this study, the information on the species with the success of sequencing the psbA-trnH sequence was extracted for the first time, the sequence length of Euphorbia thymifolia L. is 721 bp.

Figure 6 Transverse section of leaf and powder. (A) Upper epidermal cells and the stomata. (B) Lower epidermal cells and the stomata. (C) canal. (D) Nonglandular hair. (E) Mesophyll tissue (showing cells around the end of fine veins). (F) Skin epidermal cells. (G) Fiber. (H) pollen grain.

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Conclusions

In the present study, Euphorbia prostrata Ait. was identified systematically based on traditional pharmacognostical, and DNA barcoding analysis, which is beneficial to the development of the quality standard and the identification of species.

The stem of the original plant of Euphorbia prostrata Ait. branches from the base, creeps around and grows, and the whole plant is light and crisp after being dried in the shade. The transverse section of the root shows that the xylem accounts for most of the section. The pericycle fiber bundles in the transverse section of the stem are arranged intermittently and annularly, and the cortex is scattered in the latex tube. The transverse section of the leaf is scattered throughout the latex tube. The vascular bundles in the midvein have large vascular sheaths and about 20 vascular bundles. There are large cell ringlike columns around the vascular bundles in the veinlets. The stomatal indices of the upper and lower epidermis are similar, and the epidermis cells in the peel are golden yellow in color and stone-like in powder. An examination of general chemical composition showed that Euphorbia procumbens contained such compounds as saccharides, protein, flavonoids, and anthraquinones. In the powder phenomenon test, the powder all presented as sinkage, and the colors of different solvent solutions were different. The experimental results of fluorescence analysis showed that the extracts with different solvents mostly showed dark green or orange fluorescence under sunlight at 365 nm and mostly showed no fluorescence at 254 nm. The average contents of water, total ash, and acid-insoluble ash in Euphorbia procumbens were 6.13%, 16.39%, and 7.62%, respectively. The above characteristics can provide the basic basis for the crude drug identification of Euphorbia stolonifera of Euphorbiaceae.

The Lingnan region, which is close to the equator geographically, has a hot and humid climate, which is conducive to the growth of medicinal plants [10]. The resources of medicinal plants are very rich and the market is quite broad. At the same time, the confusion of Chinese medicinal varieties leads to the frequent misuse of Chinese medicinal materials. In order to solve this problem, it is necessary to study it and establish a standardized system for the quality evaluation of Chinese medicinal materials, which will provide a sufficient reference for the modernization of Chinese medicinal research.

Under the background of continuous exploitation of medicinal resources and the rapid development of contemporary Chinese medicine, the application of modern natural science knowledge and technology to the development, identification, standard setting, and other related research of medicinal plants to ensure the safety, effectiveness, stability, and controllability of Chinese medicine has become a mature subject. The comprehensive and systematic identification information of medicinal plants is of great help for the establishment of standardization of medicinal plants, follow-up research on relevant chemical components, pharmacological effects, application and promotion, etc. The species of medicinal plants in Lingnan are very complex, and their development and utilization have a very broad prospect. In this study, the four traditional identification methods combined with DNA barcode technology were systematically observed and described in detail to provide a reference for authenticity identification, genetic relationship, and further development and utilization of Euphorbia prostrata Ait.

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