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YZha was responsible for collecting plant material, identification, confection of herbarium, running the laboratory work, analysis of the data, and drafting the paper; YZhu contributed to plant collection, identification, and herbarium confection; JC contributed in the preparation of slides and analysis of data; CX, JD, HL, YL, JL, and PL designed the study, supervised the laboratory work, and read the manuscript critically

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ORIGINAL RESEARCH PAPER

Identification of three species commonly known as "daqingye" by internal leaf anatomy and high-performance liquid chromatography analyses

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Abstract
The macroscopic and microscopic morphologies and indigo and indirubin concentration of the traditional Chinese medicine herbs *Isatis indigotica* Fort., *Polygonum tinctorium* Ait., and *Baphicacanthus cusia* (Nees) Bremek, all commonly known as "daqingye", were determined and compared. The morphological analyses indicated that *I. indigotica* has leaves with winged petioles and no glandular hairs or crystals, *P. tinctorium* has leaves with membranous ocrea and clusters of calcium oxalate, and *B. cusia* has palisade cells in the mesophyll running over the main vein and single cells containing calcium carbonate crystals. Indigo and indirubin are chemical constituents that have been previously isolated from daqingye and were selected in this study as identification markers for high-performance liquid chromatography analysis due to their pharmacological activities. The chromatographic results showed that indigo and indirubin concentration varied significantly among the three species: high concentration of both indigo and indirubin were observed in *I. indigotica*, the highest concentration among the three daqingye plants was found in *P. tinctorium* but with low levels of indirubin, and the concentration of indigo and indirubin was quite low in *B. cusia*. In summary, three different species commonly known as daqingye were accurately distinguished by morphological observation, internal leaf anatomy analysis, and chromatographic analysis.

Keywords
daqingye; *Isatis indigotica*; *Polygonum tinctorium*; *Baphicacanthus cusia* (Nees) Bremek; internal leaf anatomy; high-performance liquid chromatography

Introduction

According to the World Health Organization [1], the use of traditional medicine has expanded globally over the past decade and is gaining increasing popularity. Traditional medicine continues to be used for primary healthcare of the poor in developing countries and in countries where traditional medicine dominates in the national health system. Traditional use of herbal medicines includes herbs, herbal materials, herbal preparations, and finished herbal products that contain active ingredients from plant parts. In particular, herbal food supplements are becoming increasingly popular in Western countries due to an increasing range of applications, which has further prompted an increased interest in their safety. Several studies have reported adverse effects associated with plant supplements, which can be mainly attributed to a lack of quality control [2–6]. More specifically, research has shown that adverse effects related to plant food supplements may originate from the contamination of products and the mixed use of different plant species but commonly known by the same name.

Generally, each species corresponds to a unique botanical name. However, in many cases, the same common name may be shared by more than one species [7–9]. An example of this is seen in China with leaves called "daqingye" (also known as woad leaves in English, and the literal translation of daqingye is "big green leaf" and 大青叶 in Chinese). Daqingye leaves have been used in traditional Chinese medicine for hundreds of years as hemostatic, antipyretic, antiviral, anti-inflammatory, and anti-influenza remedies [10–12]. However, the leaves of at least three different medicinal plants are commonly known as daqingye; these species include *Isatis indigotica* Fort. (Brassicaceae), *Polygonum tinctorium* Ait. (Polygonaceae), and *Baphicacanthus cusia* (Nees) Bremek (Acanthaceae) [13,14]. The same natural dye is extracted from these three plant species, which may explain the origin of the common name shared by these plants. The natural dyes indigo (also known as indigo naturalis) and indirubin are extracted from the leaves of *I. indigotica*, *P. tinctorium*, and *B. cusia*, but only the leaves of *I. indigotica* are an official source of daqingye according to the Chinese pharmacopeia [10,15]. Importantly, the water extract of daqingye plants contains the same main components found in Chinese medicinal formulae, such as pediatric antipyretic granules and cold granules for children [10].

Isatis indigotica Fort. mainly grows in Anguo of Hebei, Nantong of Jiangsu and Anhui, Shanxi, and other places. In some parts of China, people replaces *I. indigotica* with two other plants. The aerial portion of *B. cusia* is often used as an alternative to *I. indigotica* for daqingye in parts of Southern China, such as Fujian or Jiangxi provinces, and the leaves of *P. tinctorium* are often used as an alternative to *I. indigotica* for daqingye in parts of Northern China, such as Hebei or Shandong provinces [13].

Overall, more than 50 chemical constituents have been identified in daqingye plants, including alkaloids, sucrose, organic acids, and glycosides [16]. Reports have indicated that the useful contents in these plants are indigo, indirubin, tryptanthrin, and kaempferol [17,18]. Among them, we selected the two alkaloids, indigo and indirubin, as the markers for chemical composition identification purposes in this study due to their pharmacological activities (Fig. 1). Reports have indicated that indigo naturalis, prepared from leaves of plants such as *B. cusia*, *P. tinctorium*, and *I. indigotica*, has long been used to treat various inflammatory disease and dermatosis [19,20]. Indirubin, extracted from traditional herbal medicine, possesses anticancer, antileukemia effects [21,22]. Moreover, some researchers demonstrated that both indigo and indirubin have physiological effects on liver microsomes in mice [23]. Structurally, indigo is 2,2′-bisindole-3,3′(1*H*,1′*H*)-dione and it is an organic compound with a distinctive blue color. Indirubin is a purple 3,2-bisindole and a stable isomer of indigo [24].

Currently, there are several methods of analysis for determining the concentration of indigo and indirubin in plants, including spectrophotometric measurements of indigo concentration in *I. indigotica* [25,26]. Indigo and indirubin concentration can also be determined using high-performance liquid chromatography (HPLC) fingerprinting [27,28] or using liquid chromatography with atmospheric pressure chemical ionization and mass spectrometry (LC-APCI-MS) [18]. Based on a review of the available literature, we selected HPLC for a highly effective, accurate, and rapid analysis of indigo and indirubin concentration in plants.

Considering the wide geographic distribution of daqingye plants, the significant environmental differences of the producing areas, and the different local prescription, it is of practical importance to standardize the use of daqingye for medicinal purposes. However, thus far there have been no reports specifically distinguishing the three different plants commonly known as daqingye. In this study, we analyzed the macroscopic and microscopic features and two chemical components of three species commonly known in China as daqingye.

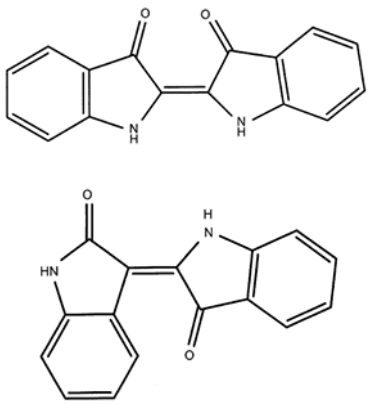

Fig. 1 Chemical structures of indigo (top) and indirubin (bottom).

Material and methods

Plant material

Seeds of *I. indigotica* and sprouts of *P. tinctorium* and *B. cusia* were purchased from Chongqing Heben Agriculture Co., Ltd. between March and May of 2014.

The seeds and sprouts were planted in May of the same year at the scientific research site of the Sichuan Academy of Agricultural Sciences in the Xindu District of Chengdu. All plant samples were identified by Professor Yingfang Wei from the Chengdu University of Traditional Chinese Medicine. Voucher specimens of all plants were properly cataloged and deposited at the School of Pharmacy, Chengdu University of Traditional Chinese Medicine, Sichuan, China. For the preparation of powdered dried leaves for research use, fresh leaves were picked from sprouts displaying abundant foliage. Fresh leaves of *I. indigotica* (collection No. 20140901), *P. tinctorium* (collection No. 20140902), and *B. cusia* (collection No. 20140903) were also used in this study. The numbers listed were the actual voucher numbers of the specimens fresh leaves, which were collected from the shoots and seeds of three plants.

Chemical reagents

Standards of indigo and indirubin were purchased from Beijing Solarbio Science & Technology Co., Ltd. The purity of these standards was analyzed using HPLC by the vendor. The *N*,*N*-dimethylformamide (DMF) used was ACS certified. Methanol, toluene, paraffin, safranine, and fast green were HPLC grade and purchased from Fisher Scientific (USA).

Internal leaf anatomy studies

Paraffin sections of leaf cross sections were prepared according to procedures previously described [29]. Paraffin sections with a thickness of 7–10 μm were prepared using a fully automatic microtome and autostainer (Leica CM1100, Leica ST5010; Leica Microsystems, Germany). The sections were dually stained with safranine and fast green. All sections were examined and photographed using an Olympus CX41 optical microscope and Toupcam industrial digital camera (UCMOSOS100KPA; Olympus, Japan).

Analysis of high-performance liquid chromatography

Handling method of standards and samples. Standard solutions of indigo (0.0048 g) and indirubin (0.0020 g) were prepared by dissolving in 5 mL DMF. Then, 450 μL indigo and 450 μL indirubin standard solutions were measured, combined, and further diluted with 900 μL DMF to prepare a mixed standard solution.

The leaves of the three daqingye plants were dried in an oven at 50°C for 5 h, crushed, and filtered (particle size: 150 μm). Two grams of each plant sample were added to 25 mL DMF and sonicated for 1 h, followed by centrifugation at 4,000 rpm for 15 min. The supernatant was transferred to a 25-mL volumetric flask. The volume was adjusted with DMF as needed so that equal volumes were used for each extract.

Before HPLC analysis, all samples were filtered through a 0.22-μm microporous membrane.

Instrument and chromatographic conditions. We used an Agilent LC 1260 Infinity HPLC system (Agilent Technologies Co., Ltd.) equipped with a G1311B 1260 quaternary pump, a G1329B 1260 automatic sampling device, a G1316A 1260 TCC column heater, a G1315D 1260 diode array detector, and a computer connected to a working station equipped with Open CDS Chemstation Edition. The chromatographic column was an Agilent Eclipse XDB-C18 (250 × 4.6 mm; packed particle size 5 μm). The column temperature was maintained at 30°C, the mobile phase comprised 70% methanol and 30% water. The flow rate was set 1.0 mL/min, the injected sample volume was 20 μL, and the sample test time was 35 min at a test wavelength of 293 nm.

Results

A detailed comparison of morphology and microscopic features is listed in Tab. 1.

Tab. 1 Detailed comparison of morphology and microscopic features of the three daqingye plants.

Sample item	*Isatis indigotica* Fort.	*Polygonum tinctorium* Ait.	*Baphicacanthus cusia* (Nees) Bremek
Color of fresh samples	Emerald green	Blue green or black blue	Gray green or dark green
Shape of leaves	Long ellipse to oblong oblanceolate shape	Oval	Long oval shape or obovate oblong
Size of leaves	5 to 20 cm long, 2 to 6 cm wide	3 to 8 cm long, 2 to 5 cm wide	8 to 15 cm long, 3 to 5 cm wide
Leaf margin	Entire or with minor undulate	Entire	Small shallow serrated
Petiole	With winged petioles around the petiole but no ocrea	Without winged petioles but with membranous ocrea	With no winged petioles nor ocrea
Midrib cross section	Palisade parenchyma does not pass over the midrib	Palisade parenchyma cells do not pass through the main vein	Palisade parenchyma cross over the main veins
	Midrib contains four to nine collateral vascular bundles; the middle vascular bundle was the largest	Five to seven vascular bundles form a circle, collateral	Single vascular bundle
	Sclerenchyma can be observed on both sides (adaxial and abaxial) of the vascular bundles	Fiber bundle in the inner side of the phloem	No sclerenchyma in the inner and outer vascular bundles
Section of leaf surface	No glandular hairs	With glandular hairs	With glandular hairs

Morphological characteristics

The leaves of *I. indigotica* were entire or had minor undulations. The apex was blunt, whereas the base part narrowed gradually to form a wing around the petiole. The petiole was 4–10 cm long (Fig. 2A). The vein was more prominent on the abaxial surface of the leaves.

The leaves of *P. tinctorium* had an elliptical or oval shape. The apex was blunt and leaf gradually narrowed towards the base. The leaves were entire and had fine serrations. The veins were elevated on the abaxial surface of the leaves. The petiole was flat with a membranous ocrea (Fig. 2B).

The leaves of *B. cusia* had a long oval or obovate oblong shape, gradually narrowing towards the base. The apex gradually narrowed towards the base. Shallow serration was present at the leaf margins (Fig. 2C).

Fig. 2 Comparison of the appearance of three daqingye plants. (**A**) *Isatis indigotica* Fort. (**B**) *Polygonum tinctorium* Ait. (**C**) *Baphicacanthus cusia* (Nees) Bremek.

Microscopic characteristics

Microscopic characteristics of the transverse section of the leaves of the three daqingye plants. The upper and lower epidermis was distinct in the cross section of the *I. indigotica* leaf. The leaf midrib was flat on the adaxial surface but slightly raised underneath. The leaves had one–two collenchyma cells on the inner side of the upper and lower epidermis; the palisade parenchyma had three–four rows of cells that were almost rectangular. The leaves contained loose spongy parenchyma (Fig. 3A,D).

The upper and lower epidermis was distinct in the cross section of the *P. tinctorium* leaf. The upper epidermal cells showed tangential extension, whereas the lower epidermal cells were smaller. The leaves were bifacial, with two–three rows of palisade parenchyma cells. Cells of spongy parenchyma were circular or ovoid. Parenchyma cells contained a large quantity of granular blue materials, and some contained large clusters of calcium oxalate. Abundant collenchyma cells surrounded the main vein protruding upwards. Outside the phloem, sclerenchyma cells were present (Fig. 3B,E).

The upper and lower epidermis was distinct in the cross section of the *B. cusia* leaf. The upper epidermal cells were rectangular or near-rectangular. The palisade parenchyma had one–two rows of cells. The spongy parenchyma was loosely arranged. The palisade and spongy parenchyma contained crystalliferous cells with round or oval-shaped crystals. Abundant collenchyma cells surrounded the main vein protruding upwards. The vascular bundles were grooved (Fig. 3C,F).

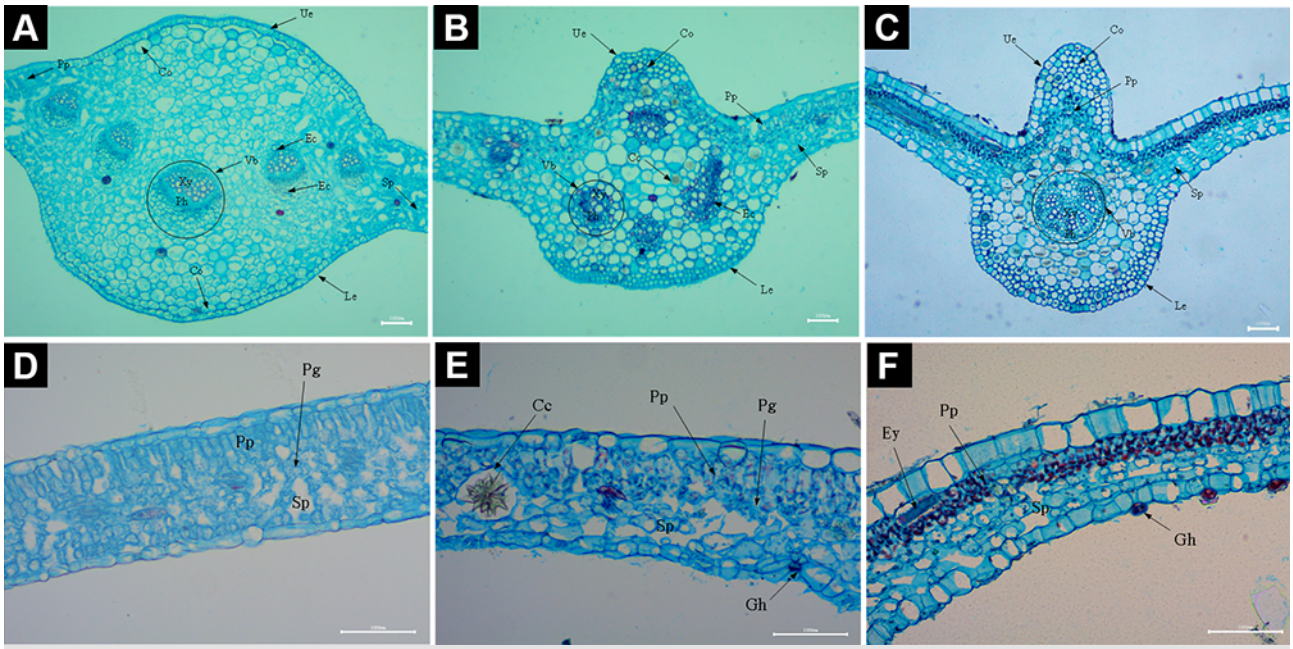

Fig. 3 Transverse sections and microscopic characteristics of leaves of the three daqingye plants. (**A**) Transverse section of *Isatis indigotica* Fort. leaves. (**B**) Transverse section of *Polygonum tinctorium* Ait. leaves. (**C**) Transverse section of *Baphicacanthus cusia* (Nees) Bremek leaves. (**D**) Local enlarged details of *I. inigotica* leaves. (**E**) Local enlarged details of *P. tinctorium* leaves. (**F**) Local enlarged details of *B. cusia* leaves. Ue – upper epidermal cells; Co – collenchyma; Pp – palisade parenchyma; Vb – vascular bundle; Sp – spongy parenchyma; Ec – sclerenchyma; Le – lower epidermal cells; Cc – clusters of calcium oxalate; Pg – pigment granules; Gh – glandular hairs; Ey – cells containing cystolith.

Surface characteristics of leaf sections of the three daqingye plants. The epidermal cell walls of *I. indigotica* were slightly rounded with anisocytic stomata and three–four subsidiary cells. The mesophyll cells contained fine blue particles (Fig. 4A). The leaf epidermal cells of *P. tinctorium* were polygonal with straight or slightly rounded in the outline. Stomata were mostly of the paracytic type. The head of the glandular hairs had four–eight cells. The mesophyll tissue contained blue or blue-black pigment granules (Fig. 4B).

Stomata were only present on the abaxial surfaces of *B. cusia* leaves. The glandular hair had short stalks, with the gland head containing four–eight cells (Fig. 4C).

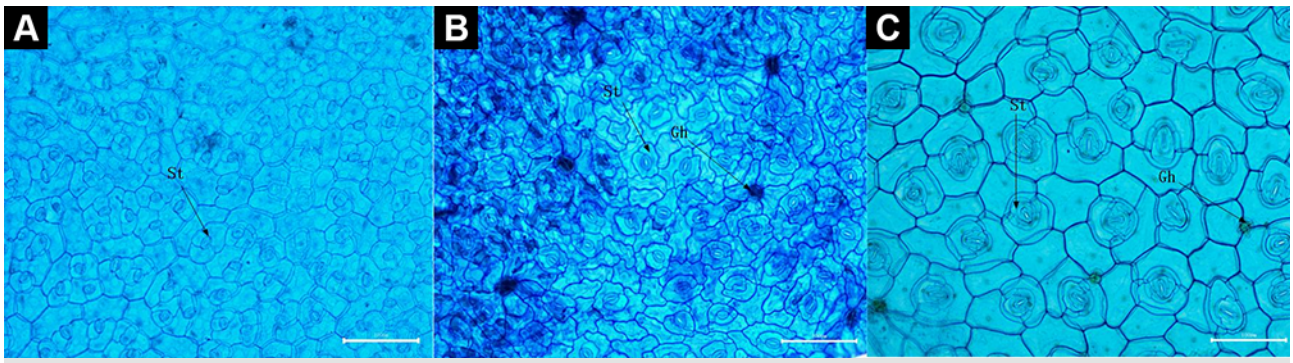

Fig. 4 Microscopic features of the lower epidermis of the leaf surface of the three daqingye plants. (**A**) *Isatis indigotica* Fort. (**B**) *Polygonum tinctorium* Ait. (**C**) *Baphicacanthus cusia* (Nees) Bremek. St – stomata; Gh – glandular hairs.

Analytical results from HPLC

HPLC was used to analyze the indigo and indirubin content differences among the three daqingye plants. The results indicated that significant differences in indigo and indirubin concentration were detected among the three daqingye plants tested. The *I. indigotica* sample showed a relatively high concentration of both indigo and indirubin, represented by the high amplitude of the chromatographic peaks. However, *P. tinctorium* showed the highest concentration of indigo among the three different daqingye plants but, conversely, low levels of indirubin. On the other hand, the *B. cusia* sample contained very low concentration of both indigo and indirubin, represented by almost undetectable chromatographic peaks. In summary, the three different daqingye plants can be easily and quickly distinguished by HPLC analysis (Fig. 5).

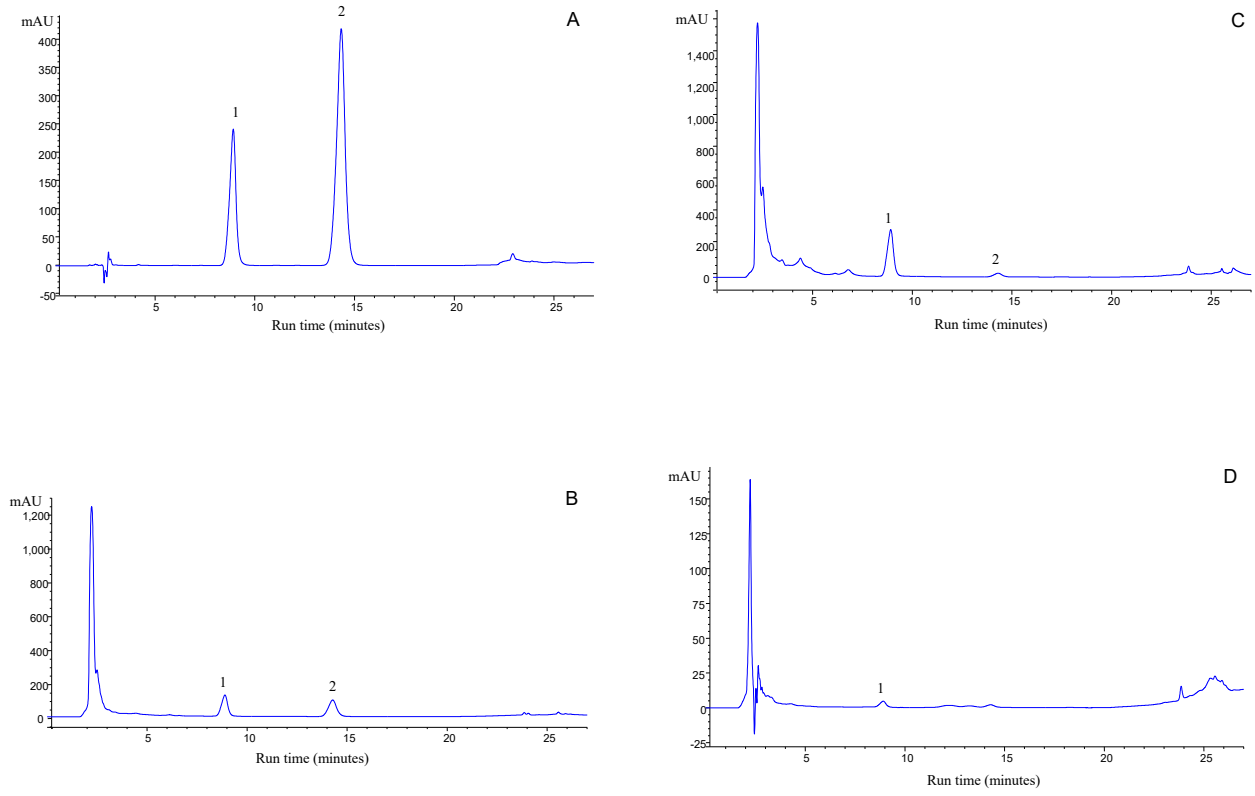

Fig. 5 High-performance liquid chromatography results of *Isatis indigotica* Fort., *Polygonum tinctorium* Ait., and *Baphicacanthus cusia* (Nees) Bremek, detected at 293 nm. (**A**) Standard mixture; (**B**) *I. indigotica*; (**C**) *P. tinctorium*; (**D**) *B. cusia*. 1 – indigo; 2 – indirubin.

Discussion

Determining the authenticity and quality of raw plant materials used in the formulation of herbal medicines is essential to ensure its safety and efficacy for clinical use. Misidentification and tampering of medicinal plant materials increases the probability of adverse events, such as the hepatotoxicity cases [3]. Yet, several raw materials are intentionally adulterated with other species that are morphologically similar, inexpensive, or more readily available [4–6].

Daqingye is a commonly used traditional Chinese medicine [30]. However, because various species are known as daqingye in China, it is of practical importance to identify and study each species to standardize the use of this herbal medicine. This manuscript aimed to characterize three plant species commonly referred to as daqingye via internal leaf anatomy and high-performance liquid chromatography analyses.

Internal leaf anatomy analysis

Identifying the internal leaf anatomy is a rapid, simple, and accurate strategy for verifying the authenticity of traditional Chinese medicines [31]. In this research, the fresh leaves of three plants known as daqingye were quickly and easily identified by comparison of morphological and microscopic features. Several anatomical and micromorphological leaf characteristics observed here can be used to identify the three plants studied: *I. indigotica* has leaves with winged petioles and no glandular hairs or crystals, the leaves of *P. tinctorium* contains clusters of calcium oxalate, and membranous ocrea, and *B. cusia* has palisade cells in the mesophyll running over the main vein and single cells containing calcium carbonate crystals.

The Chinese Pharmacopeia Commission has studied the internal leaf anatomy of *I. indigotica* and *P. tinctorium*. They reported that the epidermal cell walls of *I. indigotica* are slightly sinuous and somewhat beaded, and the stomata were anomocytic with three–four subsidiary cells. The mesophyll was indistinctly differentiated, with mesophyll cells containing numerous blue pigment granules. The epidermal cell walls of *P. tinctorium* were straight or slightly sinuous, and the stomata were mostly paracytic, with a few being anomocytic. Each glandular hair contained four–eight cells. The mesophyll tissue contained numerous blue to blue-black pigment granules, and the clusters of calcium oxalate were numerous. Overall, our observations of the microscopic features of *I. indigotica* and *P. tinctorium* leaves agreed with descriptions reported by the Chinese Pharmacopeia Commission [31].

The leaves showed remarkable interspecific anatomical variation. Nevertheless, we suggest that further studies are necessary to identify the anatomical patterns of the flowers to further provide distinct descriptions of each plant.

Chemical composition analysis

Traditional Chinese medicinal materials are usually dried and crushed before use. It is not possible to observe the macroscopic structure of crushed leaves with any precision, and no vascular bundles could be observed in the powdered daqingye leaves used here. Thus, neither external morphology nor internal leaf anatomy could be analyzed on the dried leaves, which are the final medicinal product. To address this challenge, chemical analysis methods such as HPLC can be used. In this regard, the combination of microscopic and HPLC analyses renders the plant species identification process more reliable and dependable [32,33].

From the leaves or the aerial part of *P. tinctorium*, *I. indigotica*, and *B. cusia*, indirubin and indigo were isolated as two major constituents [11]. Indigo and indirubin were previously selected as identification markers for HPLC analysis of *I. indigotica* due to their pharmacological activities [18]. Similarly, we selected indigo and indirubin as the markers for this study. Accordingly, HPLC analysis exhibited significant differences in the concentration of indigo and indirubin among the three daqingye plants studied. *Isatis indigotica* possessed relatively high concentration of both indigo and indirubin, which was represented by the high-amplitude chromatographic peaks. However, *P.*

tinctorium was found to have the highest concentration of indigo among the three different plants but extremely low concentration of indirubin. Both indigo and indirubin were barely detectable in *B. cusia*. Our *P. tinctorium* results are in accordance with a study from other workers [28] who evaluated the indigo and indirubin concentration of *P. tinctorium* leaf samples from three different sources and found the indigo concentration to be much higher than that of indirubin.

Previous studies have shown indigo and indirubin to be the main effective components of three species [13–19], but our research showed large differences in the concentrations of indigo and indirubin among these species. They have similar pharmacological activity, so this may be because of the differences in testing samples. Different individuals of the same species may differ in their chemical constituents. There may also be other similar pharmacological activity in plant effective components. This merits further study.

Overall, to the best of our knowledge, this study represents the first analysis of the leaves of *B. cusia* using a combination of internal leaf anatomy and HPLC analyses. We expect that our findings will provide guidance for proper usage of daqingye and a basis for further scientific research.

Conclusion

In this paper, we described the macroscopic and microscopic features and indigo and indirubin concentration of three different plant species commonly known in China as daqingye. Based on our findings, the three daqingye plants studied can be differentiated by internal leaf anatomy analysis and chemical analysis of major constituents.

Acknowledgments

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