Pharmacognostic Identification of Lonicera fulvotomentosa (Post-print)

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Abstract

Lonicera fulvotomentosa possesses multiple pharmacological effects including anti-inflammatory, antibacterial, and immunomodulatory activities; however, it is easily confused with other species within the Lonicera genus. Pharmacognostic studies will provide a scientific basis for the identification of Lonicera fulvotomentosa and the establishment of medicinal material standards. This study employed botanical identification, microscopic observation, thin-layer chromatography (TLC) identification, and molecular identification methods to conduct exclusive characteristic identification of Lonicera fulvotomentosa from aspects of botanical traits, microscopic features of the medicinal material, TLC profiles, and ITS (internal transcribed spacer) sequence characteristics. The results demonstrated: (1) The transverse section of Lonicera fulvotomentosa flowers contained numerous secretory cells, with yellow-brown glandular trichomes on the outer surface of the petals; the upper layer of corolla epidermal cells was polygonal, pollen sacs were clam-shaped and dehiscent, pollen grains were regularly shaped with two types—triangular and oval—and oil chambers were elliptical. (2) Powder microscopic examination revealed that pericyclic fibers were short and fusiform; cork cells had distinct angular edges and were light yellow in color; wood fibers were thick and short fusiform, occasionally curved; reticulate vessels were abundant, with calcium oxalate prisms densely distributed within the cell lumina. (3) Thin-layer chromatography showed that the content of kaempferol was relatively high in Lonicera fulvotomentosa flowers, and kaempferol could serve as a marker component for Lonicera fulvotomentosa. (4) Phylogenetic clustering results based on ITS sequences demonstrated that ITS sequences could serve as a DNA barcode to accurately differentiate Lonicera fulvotomentosa, Lonicera macranthoides, Lonicera confusa, and Lonicera japonica. These results indicate that Lonicera fulvotomentosa possesses distinct pharmacognostic characteristics, and this study provides a reference for the identification, component analysis, and quality standard establishment of Lonicera fulvotomentosa medicinal materials.

Full Text

Preamble

Pharmacognostic Identification Study of *Lonicera fulvotomentosa*

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Abstract

Lonicera fulvotomentosa possesses multiple pharmacological effects including anti-inflammatory, antibacterial, and immunomodulatory activities, but it is easily confused with other *Lonicera* species. Pharmacognostic research on this species provides a scientific basis for its identification and the establishment of medicinal material standards. This study employed botanical identification, microscopic observation, thin-layer chromatography (TLC), and molecular identification to characterize the specific traits of *L. fulvotomentosa* from multiple perspectives including botanical characteristics, microscopic features of medicinal materials, TLC profiles, and ITS (internal transcribed spacer) sequence characteristics. The results demonstrated: (1) The flower cross-section contained numerous secretory cells, with yellow-brown glandular hairs on the petal outer surface. The corolla epidermal cells were polygonal, pollen sacs were mussel-shaped and dehiscent, pollen grains were regularly shaped (triangular or oval), and oil chambers were elliptical. (2) Powder microscopy revealed short prismatic pericycle fibers; sharply angular, light-yellow cork cells; thick, short prismatic wood fibers occasionally showing curvature; abundant reticulate vessels; and cell cavities densely packed with calcium oxalate prisms. (3) TLC analysis showed high kaempferol content in *L. fulvotomentosa* flowers, suggesting kaempferol could serve as a marker compound for this species. (4) Phylogenetic clustering based on ITS sequences indicated that ITS can function as a DNA barcode to accurately distinguish *L. fulvotomentosa* from *L. macranthoides*, *L. confusa*, and *L. japonica*. These results confirm that *L. fulvotomentosa* possesses distinct pharmacognostic characteristics, providing a reference for its identification, constituent analysis, and quality standard formulation.

Keywords: *Lonicera fulvotomentosa*, microscopic features, TLC identification, ITS cluster analysis

Introduction

Lonicera fulvotomentosa Hsu et S. C. Cheng is a liana belonging to the Caprifoliaceae family, primarily distributed in Yunnan, Guizhou, and Guangxi provinces of China. Its nutritional and chemical composition is similar to that of *Lonicera japonica* Thunb., and it is commonly used as a substitute for *L. japonica* with demonstrated efficacy in preventing and treating influenza, SARS, and coronavirus infections. Previous studies have shown that flower buds of *Lonicera* species contain various bioactive compounds including phenolic acids, flavonoids, triterpenoid saponins, and alkaloids. The caffeoylquinic acid derivatives in *L. japonica* exhibit antitumor and anti-HIV activities, and research suggests that active components from *Lonicera* medicinal plants hold excellent potential for treating and preventing human refractory diseases and epidemic illnesses.

Although both *L. fulvotomentosa* and *L. japonica* (commonly known as honeysuckle) belong to the same genus, their chemical compositions differ significantly. The 2015 edition of the Chinese Pharmacopoeia establishes different standards for their main constituent contents. Traditional Chinese medicine practitioners habitually substitute *L. fulvotomentosa* for honeysuckle, necessitating accurate identification methods to avoid adverse consequences from misuse. While comparative studies have been conducted on morphological, microscopic, spectroscopic, physicochemical, and biotechnological identification between *L. macranthoides* and honeysuckle, the rich content of phenolic acids, flavonoids, and triterpenoid saponins in *Lonicera* flower buds makes species differentiation based solely on chemical composition challenging. Current pharmacognostic reports on *L. fulvotomentosa* lack detail and are impractical for identification work. Therefore, comprehensive pharmacognostic characterization and establishment of multiple complementary identification techniques are essential for quality control, efficacy assurance, and clinical application of this medicinal material.

Materials and Methods

1.1 Medicinal Material

Samples were collected from Zerong Town, Xingyi City, Guizhou Province, and identified as *Lonicera fulvotomentosa* flowers and flower buds by Researcher Wang Xujun from the Hunan Academy of Forestry. Voucher specimens are deposited in the Herbarium of the College of Biological and Food Engineering at Huaihua University.

1.2 Instruments and Reagents

Instruments: COSUAI grinder (Hunan Jide Electric Appliance Co.), Kelong 101 constant temperature drying oven (Huaihua Keyi Co.), BSM electronic balance (Shanghai Jingping Instrument Co.), HH-1 constant temperature water bath (Changsha Keyi Co.), VILBER INFINITY 3026 gel imaging system

(Shanghai Zhicheng Instrument Co.), YH/TDL-5000bR refrigerated centrifuge (Sichuan Zhiyan Technology Co.), 101-0 electric thermostatic blast drying oven (Shanghai Yaoshi Instrument Equipment Factory), API-258 rotary microtome (Hangzhou Aipu Equipment Co.), XSP-201B biological microscope (Shanghai Puqian Optical Instrument Co.), and 3PrimeX thermal cycler (Techne, UK).

Reagents: Iodine, potassium iodide, safranin, formaldehyde, acetic acid, hematoxylin stain, paraffin, glycerin, PCR reagents (Taq polymerase, dNTPs), and TBE buffer.

2.1 Macroscopic Identification

The morphological characteristics of *L. fulvotomentosa* stems, leaves, and flowers were observed, including color, shape, size, texture, and fracture features of fresh medicinal materials.

2.2 Microscopic Identification

Following the thin-layer chromatography method (General Rule 0502, Part IV, Chinese Pharmacopoeia 2020 edition), fresh flowers and flower buds were collected in ampoules, treated with hematoxylin stain, and incubated at 40°C for 24–36 hours. After rinsing, samples were soaked in dilute ammonia solution (1–2%) for 15 minutes, then dehydrated sequentially with 35%, 55%, 75%, and 95% ethanol. Following 4–6 minutes of xylene immersion, specimens were embedded in paraffin overnight. Embedded samples were trimmed, mounted on wooden blocks, sectioned, fixed with gelatin, and dried on a slide warmer. After dewaxing and counterstaining with safranin prepared in 95% ethanol, slides were observed under a biological microscope.

2.3 Thin-Layer Chromatography Identification

Following the TLC method (General Rule 0502, Part IV, Chinese Pharmacopoeia 2020 edition), flowers, young leaves, and young stems of *L. fulvotomentosa* were pulverized and sieved through a 40-mesh screen. One gram of each powder was extracted with 20 mL of 50% ethanol by refluxing in a water bath for 30 minutes. After cooling in ice water and filtration, the filtrate was evaporated to dryness. The residue was dissolved in 4 mL methanol for analysis. A reference solution of kaempferol (0.50 mg $\cdot$ mL^{-1}) was prepared in methanol. Sample and reference solutions were spotted on the same silica gel G plate and developed with ethyl acetate-formic acid-water (8:1:1) at 25°C and 65% relative humidity. After spraying with 10% sulfuric acid in ethanol and drying, plates were examined under 365 nm UV light.

2.4 Molecular Identification

Total DNA was extracted from young leaves, mature leaves, and flowers of *L. fulvotomentosa* using a modified CTAB method as described by Jiang et

al. (2010). Primers were designed as ITS-F: GGAAGGTAAAAGTCAAGG and ITS-R: TCCTCCGCTTATTGATATGC. PCR amplification followed the protocol of Jiang et al. (2010). DNA fragments were recovered, cloned into pMD 18-T vector, and six positive clones were selected for plasmid extraction and sequencing, with direct sequencing of PCR products also performed. Complete ITS1 and ITS2 sequences of *Lonicera* species were downloaded from the NCBI database. Phylogenetic trees were constructed using the unweighted pair-group method with arithmetic means (UPGMA) in MEGA X software.

Results and Analysis

3.1 Macroscopic Identification

Lonicera fulvotomentosa is a perennial climbing plant with yellow-brown pubescence on stems and branches, occasionally with orange-red glandular hairs on young shoots. Young bud scales typically occur in four pairs. Leaf shape varies from needle-like to elliptical during development, with color changing from yellow-brown to green. Mature leaves measure 4.0–7.0 cm in length and 1.5–3.0 cm in width, with petioles 4.0–7.0 cm long densely covered with yellow-brown glandular hairs. The leaf blade is narrowly elliptical with yellow-brown glandular hairs on both surfaces and reticulate venation, with prominent glandular hairs along the midrib. Leaf spacing is 1.0–1.5 cm at stem apices and up to 6.0 cm on lower portions. Short racemes arise from leaf axils or young branch apices. Bracteoles are long subulate, 4.0–7.0 mm in length, with dense, short, stiff glandular hairs. The calyx tube is ovate-elliptical, 1.0–3.0 mm long, glabrous, with calyx teeth longer than the tube. Flowers are white in early stage, turning yellow later. Open flowers measure 3.0–5.0 cm in length, with corolla tubes shorter than lips, covered externally with short yellow-brown glandular hairs. Filaments and styles are relatively long, with exposed anthers and stigma surfaces that are smooth and glabrous. The stigma is short-elliptical. Internodes are short, typically bearing 10–22 flower clusters that bloom relatively synchronously. The flowering period is brief, lasting approximately two weeks. Berries are spherical and black.

3.2 Microscopic Identification of Flower Cross-Sections

The corolla epidermal cells of *L. fulvotomentosa* showed chain-bead thickening of anticlinal walls and wavy striations on the outer periclinal walls. Pollen sacs were plump, mussel-shaped, and bilaterally dehiscent, measuring 310.0–370.0 m in diameter. Petal epidermal cells were elongated and tightly arranged with long strip-like wall thickening. Numerous sharp-angled calcium oxalate cluster crystals were present in anticlinal walls. Petal parenchyma cells were nearly circular, 4.0–12.0 m in diameter. Pollen grains were yellowish, triangular or long oval, 5.0–15.0 m in diameter, with distinct germination furrows. Oil chambers were short-elliptical with abundant secretory cells in cross-section, accompanied by yellow-brown secretions and unicellular non-glandular hairs nearby. Details are shown in Figure 1 [Figure 1: see original paper].

3.3 Powder Microscopic Characteristics

Pollen powder appeared yellow, unicellular and spherical, occasionally pale yellow-green. Pericycle fibers were short prismatic, 19.0–47.0 μm in diameter with thin walls (2.0–7.0 μm thick). Cork cells were sharply angular, light yellow, and numerous. Wood fibers were thick and short prismatic, occasionally curved, occurring in bundles or scattered, 5.0–52.0 μm in diameter with wall thickness of 2.0–14.0 μm and distinct oblique slit-like pits. Lignified parenchyma cells contained abundant compound starch grains. Bordered-pitted and reticulate vessels were abundant, approximately 25.0–50.0 m in diameter. Crystal-containing cells showed obvious lignified wall thickening with numerous calcium oxalate prisms in cell cavities. Resin ducts contained scattered yellow-brown secretions that were translucent.

3.4 Thin-Layer Chromatography Identification

Flower extracts from *L. fulvotomentosa*, *L. confusa*, *L. japonica*, and *L. macranthoides* were spotted alongside kaempferol on the same TLC plate and examined under 365 nm UV light. As shown in Figure 3 [Figure 3: see original paper], kaempferol and all four *Lonicera* species extracts exhibited strong light blue fluorescence at identical positions. *L. japonica* and *L. macranthoides* showed four bands, *L. confusa* showed three bands, while *L. fulvotomentosa* displayed only two bands, with the kaempferol band being more distinct than in the other three species. These results indicate high kaempferol content in *L. fulvotomentosa*, and demonstrate that kaempferol TLC banding patterns can differentiate this species from *L. confusa*, *L. japonica*, and *L. macranthoides*.

3.5 Molecular Identification

As shown in Figure 4 [Figure 4: see original paper], PCR amplification using genomic DNA from young leaves, mature leaves, and flowers of *L. fulvotomentosa* yielded bands of identical size (615 bp). The obtained ITS sequences were assembled, verified, and confirmed through BLAST comparison in the NCBI database. Seventeen *Lonicera* species ITS sequences (600–620 bp) containing complete ITS1 and ITS2 regions were downloaded from NCBI. UPGMA phylogenetic analysis (Figure 5 [Figure 5: see original paper]) revealed two major groups: one comprising the medicinal species *L. macranthoides*, *L. fulvotomentosa*, and *L. confusa* (collectively known as "Shanyinhua") plus *L. japonica*, and another containing other *Lonicera* species, both with 100% bootstrap support. The analysis further showed close genetic relationships among *L. fulvotomentosa*, *L. macranthoides*, and *L. confusa*, supporting their classification within the Shanyinhua group. These results demonstrate that ITS sequences can serve as a DNA barcode to distinguish *L. fulvotomentosa* from *L. macranthoides*, *L. confusa*, and *L. japonica*.

Discussion and Conclusion

In recent years, traditional Chinese medicine practitioners have paid limited attention to the selective use of specific medicinal species or varieties and their chemical constituents, often treating herbal medicines as natural chemical mixtures and roughly assuming that several medicinal species within the same genus are interchangeable. However, congeners with different chemical compositions may exhibit distinct pharmacological effects, and different components may act synergistically or antagonistically, leading to significant variations in therapeutic efficacy. Identifying the botanical sources of Chinese medicinal materials represents an urgent concern. The complexity of active constituents and factors affecting their content variation poses a major bottleneck for quality identification and evaluation of traditional Chinese medicines, necessitating the establishment of unique and specific detection technologies and quality standards for different medicinal materials. In recent years, continuously improved TLC technology has become a relatively inexpensive and user-friendly tool for natural product chemical identification and analysis, particularly with the development of high-performance thin-layer chromatography (HPTLC), which enables simple, rapid simultaneous detection of multiple components and shows extreme sensitivity for flavonoids such as quercetin and kaempferol. The prerequisite for promoting and applying TLC technology in medicinal material detection is identifying unique and specific marker compounds for each material, which can be achieved through pharmacognostic research.

Lonicera medicinal plants demonstrate specific efficacy in treating exogenous wind-heat, epidemic febrile diseases, and infectious conditions such as sores and scabies. Their antibacterial, anti-inflammatory, and antioxidant components are closely related to their unique pharmacological activities. The genus *Lonicera* comprises numerous medicinal species, including 2 subgenera, 4 sections, 14 subsections, and 200 species. The five *Lonicera* species recorded in the Chinese Pharmacopoeia show similar flower bud morphology and are easily confused, yet their chemical compositions differ, resulting in distinct clinical usage and dosages. Since the 2015 Pharmacopoeia edition, Shanyinhua (*L. macranthoides*, *L. confusa*, *L. fulvotomentosa*, and *L. hypoglauca*) have been considered common adulterants of honeysuckle (*L. japonica*). However, due to varying usage habits and methods among Chinese and ethnic folk medicine practitioners, different species are employed in treating different diseases. Comprehensive pharmacognostic identification of *Lonicera* medicinal plants using multiple techniques is crucial for improving therapeutic efficacy.

This study conducted comprehensive pharmacognostic identification of *L. fulvotomentosa* from macroscopic to microscopic levels, combining conventional botanical identification, microscopic observation, TLC, and molecular methods. Yang et al. (2007) identified two types of non-glandular hairs (thin-walled and thick-walled) in *L. fulvotomentosa* flower buds through powder microscopy. Building upon this, our study detected secretory cells containing yellow-brown secretions in the powder, which may be related to the color of the medicinal

material. Previous TLC detection found that although Guizhou-produced *L. macranthoides* and *L. fulvotomentosa* showed fluorescent spots at the same position as chlorogenic acid reference, the similar spot numbers and sizes made them unsuitable for differentiating *Lonicera* species. Our TLC results demonstrate that kaempferol appears as a clear, high-content band in *L. fulvotomentosa*, while *L. confusa*, *L. japonica*, and *L. macranthoides* show two or more additional spots besides kaempferol (with specific components yet to be identified). We propose that kaempferol can serve as an important marker compound for TLC detection of *L. fulvotomentosa*, representing a flavonoid distinct from the characteristic honeysuckle compounds chlorogenic acid and luteolin.

With the advent of the post-genomic era, obtaining large-scale genomic data for *Lonicera* medicinal species has become more feasible, and molecular systematics and genomics research may reveal the quality formation mechanisms of this genuine medicinal material. DNA barcoding can accurately reflect evolutionary relationships and shows great potential for species identification. Traditional Chinese medicines are typically extracts or dried raw materials from which DNA extraction is difficult, and shorter PCR products are easier to amplify. The ITS2 region of ribosomal DNA, typically approximately 260 bp in length, has been identified as a suitable DNA barcode region with high interspecific divergence and intraspecific conservation. Han et al. (2013) used ITS2 to identify honeysuckle from *Lonicera* plants, but the amplified ITS2 was shorter than expected. Moreover, since most Chinese medicines are compound formulas involving multiple medicinal materials, identification using a single pair of ITS2 universal primers is not ideal for dozens of herbal components, posing significant obstacles for commercial applications. In this study, we obtained ITS sequences of 615 bp from fresh *L. fulvotomentosa* leaves and flowers. ITS sequence lengths from NCBI were 617 bp for *L. japonica*, 613 bp for *L. confusa*, and 613 or 610 bp for *L. macranthoides*. Phylogenetic analysis revealed that among *Lonicera* medicinal species, *L. fulvotomentosa*, *L. macranthoides*, and *L. confusa* clustered into one major group, while *L. japonica* formed another group. These findings indicate that ITS sequence-based studies on genetic and evolutionary mechanisms of *Lonicera* medicinal plants facilitate their classification and identification, and that ITS sequences can serve as a DNA barcode to differentiate these four *Lonicera* species.

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Note: Figure translations are in progress. See original paper for figures.