Application of microscopic techniques for the authentication of herbal medicines

Zhongzhen Zhao
School of Chinese Medicine, Hong Kong Baptist University, Kowloon, Hong Kong

Microscopic identification is the method most commonly used to authenticate Chinese herbal medicines. By means of various microscopic techniques, structural and cellular features of herbs are examined in order to determine their botanical origins and assess their qualities. This method is useful for identifying species from fragments or powders and for distinguishing species with similar morphological characters; it may also be useful for evaluating the pharmaceutical quality of herbs. In this chapter, techniques of preparing specimens for microscopic examination are described. Normal light microscopy combined with polariscope and fluorescence microscopy are introduced as they are used in the authentication of Chinese herbal medicines. Structures and cells of herbs have been found to possess stable and specific polariscopic and fluorescent characteristics that can be used to determine the botanical identity of herbal medicines. Furthermore, ordinary light microscopy is still the most practical method for primary authentication. It has been used in identifying species from fragments or powders and for distinguishing species with similar morphological characters; it may also be useful for evaluating the pharmaceutical quality of herbs. In this chapter, techniques of preparing specimens for microscopic examination are described. Normal light microscopy combined with polariscope and fluorescence microscopy are introduced as they are used in the authentication of Chinese herbal medicines. Structures and cells of herbs have been found to possess stable and specific polariscopic and fluorescent characteristics that can be used to determine the botanical identity of herbal medicines. In addition, the future prospect of combining microscopes with chemical analysis to analyze the chemical profiles of herbal tissues is discussed. Establishing a correlation between microscopic features and active components distributed in a plant’s tissues will enable quality evaluation of Chinese herbal medicines by microscopy.

Keywords Chinese herbal medicine; authentication; polariscope; fluorescence microscope

1. Introduction

Herbal medicines are being used more and more widely throughout the world. Among these, Chinese herbal medicines (CHMs) are among the most popular. This trend poses two new problems, with international implications, which increase the importance of fast, accurate, and efficient means of authenticating herbs. First, the growing market for CHMs worldwide has spawned many CHMs trading companies and generated an increase in not only counterfeit herbs but also herbs of questionable quality. Second, Chinese herbal medicines differ in significant ways from their Western herbal counterparts, raising different problems of quality control and traceability. CHMs are often processed. That is, the crude drug may undergo a series of treatments that render it chemically different from the source drug. Second, CHMs are often taken as complex prescriptions of ten, twenty or even thirty different components. These unique features of CHMs generate equally unique problems of authentication, such as determining if there is species confusion of different herbs sharing one name or one herb using different names and if the correct CHMs have been included in a particular proprietary medicine. As poisoning incidents have been caused by misuse or confusion of CHMs, their safe use has raised international concern, and authentication of CHMs is critical to their safe and effective use [1].

Today, there are a variety of methods available to authenticate CHMs, ranging from simple morphological examination to physical and chemical analysis, and DNA molecular biology. Each method has drawbacks and advantages. In difficult or critical cases, sometimes two or several methods are applied for the authentication of CHMs. Nevertheless, ordinary light microscopy is still the most practical method for primary identification. It has been commonly used in the authentication of herbal medicines in China and many other countries because of its virtues of small amount of sample needed, fast speed and low cost. Furthermore, herbal medicines are mostly low cost medicine, which should not be raised in price as a consequence of the application of unnecessary highly sophisticated methods for authentication. In China it has been used in the authentication of CHMs since the 1953 edition of the Chinese Pharmacopoeia was published [2]. Many reference books of CHMs still record the microscopic characteristics of each CHM, e.g., Chinese Materia Medica [3] and New Compendium of Chinese Materia Medica [4]. Outside China, the pharmacopoeias of other countries also record the microscopic characteristics of their herbal medicines, for examples, European Pharmacopoeia [5], British Pharmacopoeia [6], United States Pharmacopeia [7], Japanese Pharmacopoeia [8], Ayurvedic Pharmacopoeia of India [9] and Vietnamese Pharmacopoeia [10].

Using the microscope to determine the identity of herbal medicines, namely, microscopic authentication, refers to observing cell structure and internal features using a microscope and its derivatives. Besides the ordinary light microscope, other microscopes also have been used to enhance the accuracy of authentication, such as the polarized light microscope and fluorescence microscope. Use of these microscopes expands the number of features available for use in identification. For example, it has been found that starch grains, crystals of calcium oxalate, stone cells, vessels and fibres have stable and special polariscopic characteristics [11, 12]. The fluorescence microscope reveals the fluorescence emitted from herbal tissues under illumination. Many herbal tissues, by virtue of their chemical structures or secondary metabolites, have the ability to emit light of a specific wavelength following the absorption of light with a shorter wavelength and higher energy [13]. For example, in recent years, the fluorescence microscope has been applied for distinguishing the medicinal herb Oldenlandia diffusa from other species of the same genus which are confused with
it in herbal markets [14]. Elsewhere, the fluorescence microscope and microspectromter have been used to authenticate four kinds of powdered CHMs and measure the distribution of chemicals in the cross sections of CHMs [15].

Recent innovations in normal light microscopy have greatly enhanced its usefulness in the authentication of herbal medicines. Feature extraction and similarity measurement as well as the use of chord length distribution have been used effectively in the classification of starch grains in microscopic images of CHMs. This provides greater accuracy and flexibility in capturing information about starch grains which are useful in authentication of CHMs [16, 17]. A novel method which combines histological and microscopic analysis of laticifers by "blob" analysis has been established to distinguish species of Radix Fici (Wuzhimaotao in Chinese name) which were otherwise difficult to distinguish by microscopic examination; the method provides objective data to describe and standardize the characters observed in microscopic images [18].

Microscopic techniques have been widely and effectively applied for the authentication of herbal medicines. In this chapter, the standard procedures and application of microscopic identification will be described, and the future prospects for this work will be discussed.

2. Standard Procedure for Microscopic Identification

In microscopic identification, the microscope is used to examine transverse or longitudinal sections, powder, surfaces or disintegrated tissues of crude drugs and/or of Chinese proprietary medicines mounted on glass slides.

2.1 Sampling

The validity of sampling directly affects the accuracy of identification results; therefore reliable, random procedures of sampling should be strictly followed. In the description below, “R.S.” refers to reference samples, while “T.S.” refers to test samples.

R.S. are essential for microscopic identification. The standard R.S. should be determined after strict botanical taxonomy identification of the original plant. Apart from accurately identifying the original plants of CHM, it should be noted that the microscopic features of the test object might show variation by growth period and environment. Therefore the production place, collection time and processing methods should be recorded.

For T.S., origins, production place, specification, grade, packaging style should be noted, and integrity of package, hygienic level, water trace, extent of mildew and rot and contamination with foreign matter should also be checked and recorded in detail.

The average quantity of samples should be no less than 3 times what is required for testing. 1/3 of the sample is used for experimental analysis, 1/3 is used for verification while the remaining 1/3 is retained for at least a year.

2.2 Making Specimen Slides

The quality of specimen slides is critical to microscopic identification; good quality is essential. The method of making slides should be chosen according to the nature of the material at hand, and the purpose of the investigation.

2.2.1 Transverse Sections

There are four main methods for mounting transverse sections, namely, freehand mounting; glide mounting; cryology mounting; and paraffin mounting. In all cases, all slides should be clearly labelled with identifying information, e.g., name, serial number, origin, date.

(1) Freehand mounting: This method is mainly for making temporary slides. The general procedure for making a freehand cross-section is as follows: Hold the specimen in the left hand; with a sharp blade in the right hand, put the blade against the material and slice smoothly from the upper left toward the lower right in a single motion. Avoid sawing back and forth; keep the specimen and blade lubricated with water.

(2) Glide mounting: This method, using a gliding mounting machine, is suitable for lignum, ligneous roots, stems or other solid materials. Gliding mounting machine, named as sliding microtome, mainly is composed of specimen feed, knife holder and specimen orientation. The sturdy construction gives it qualities that ensure excellent, reproducible sectioning results. The section thickness and knife inclination and declination can be adjusted.

(3) Cryology mounting: This method is mainly used to make slides of animal tissue, fresh and young herbal tissue. The steps are as follows: Cut the sample into small pieces (about 1-2 cm in diameter) and embed them with cryomatrix on a cryocasste; freeze them; slice using a machine; mount on glass slides; seal.

(4) Paraffin mounting: This method entails embedding specimens in paraffin, then slicing the block. The steps include sampling, fixing, dehydration, vitrification, olefin immersion, olefin embedding, slicing, removing the paraffin, staining with, e.g., safranin and fast green, vitrification after replacing the dyeing solution with a low to high gradient concentration of ethanol, and finally sealing the mounted specimen with gum arabic or neutral gum.
2.2.2 Powders

(1) Pre-treating: The cleaned sample is pulverized. The powder should pass through a No. 4 sieve (average internal diameter of aperture: 250 ± 9.9 µm) to obtain fine granules.

(2) Mounting: Place the powdered material on the slides. Add 1-3 drops of testing agents; if necessary stir with a fine pointed needle to distribute testing agent evenly. Add cover slip. Remove any excess liquid that may exude from under the cover slip by blotting around its edges gently with filter paper.

2.2.3 Fragments

In the case of pieces of tissue, place them on a slide, add wetting agent, tease apart with dissecting needles. Then add a cover slip.

2.3 Photography

In order to make an accurate and useful record of microscopic features, using the microscope and skills of photographing are very important. Digital methods of storage make it more and more convenient to save and share suitable pictures. Setting the functions of exposure time and contrast, crop selection, microscopic measuring must be mastered.

3. Identification of Chinese Herbal Medicines

3.1. Applications

Microscopic identification has an extensive application in identification of CHMs for determining confused species, counterfeits and the identity of ingredients of Chinese proprietary medicines. The use of polarized technique and fluorescence microscope ensures a more accurate and faster identification, which further expands the application. In recent years, many studies on the authentication of herbal medicine by microscopic techniques have been reported, for examples, authentication of toxic and potent Chinese Materia Medica [19, 20]; authentication of traditional Tibetan herbal medicine of *Halenia elliptica* D. Don and “Meiduoluomi” from eight *Aster* species [21, 22]. The following are some examples of how the technique has been successfully used.

(1) Identification of herbal tea—“Ku-Ding-Cha” [23]

Ku-Ding-Cha, a kind of herbal tea, has been widely used in China for a long time to support cardio-vascular health. Confusion arises because in different parts of China different plants are used to produce the commercial teas. A comparative study was made on 24 samples of Ku-Ding-Cha including five standard identified and authenticated plants and nineteen commercial samples by microscopic techniques (Table 1 and 2, Fig. 1). The results showed that the shapes of leaf blades, xylem cells, stone cells and calcium oxalate crystals could be used to identify the plants from which the commercial products were made.
Table 1 The Microscopic Characteristics of Leaf Transverse Sections (Sketch) and Powder of Five Standard Original Plants

<table>
<thead>
<tr>
<th>No.</th>
<th>Latin Name</th>
<th>Location</th>
<th>Transverse section</th>
<th>Powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>Ilex kudingcha</em> C. J. Tseng</td>
<td>Guangxi, China</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>B</td>
<td><em>Ilex latifolia</em> Thumb.</td>
<td>Guangxi, China</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>C</td>
<td><em>Ehretia thyrsiflora</em> (Sieb. et Zucc.) Nakai</td>
<td>Guangxi, China</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>D</td>
<td><em>Clerodendrum fortunatum</em> L.</td>
<td>HongKong, China</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td>E</td>
<td><em>Ligustrum robustum</em> (Roxb.) Bl.</td>
<td>Yunnan, China</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
</tr>
</tbody>
</table>

* A1, A3, B1, B3, B5, C1, C3, D1, D2, E1, E2, E3 were observed under normal light microscope; A2, A4, B2, B4, B6, C2, C4, E4 were observed under polarized light microscope.
Table 2 Key elements of microscopic identification for Ku-Ding-Cha

**(Transverse section)**

1. No pith in the centre
2. No non-glandular hairs on the upper surface
3. Xylem heart-shaped .................................................A) *Ilex kudingcha* C. J. Tseng
5. With pith in the centre
7. With pith in the centre
8. No non-glandular hairs on the upper surface...........................C) *Ehretia thyrsiflora* (Sieb. et Zucc.) Nakai
9. Non-glandular hairs on the upper surface.........................B) *Clerodendrum fortunatum* L.

**(Powder characteristics)**

1. Presence of stone cells
2. Stone cells subrounded or subsquare ......................... A) *Ilex kudingcha* C. J. Tseng
3. Stone cells irregularly shaped; and presence of prisms of calcium oxalate, polyhedral, rectangular in shape; 15~25µm in diameter.......................................................... D) *Ilex latifolia* Thumb.
4. Presence of clusters of calcium oxalate, several arranged in rows, commonly with sharp angles; 30~50µm in diameter.........................................................C) *Ehretia thyrsiflora* (Sieb. et Zucc.) Nakai
5. Presence of prisms of calcium oxalate, fine and abundant, consisting in parenchymatous cells; and presence of non-glandular hairs...................... E) *Ligustrum robustum* (Roxb.) Bl.
6. No calcium oxalate................................................. B) *Clerodendrum fortunatum* L.

(2) Distinguishing the medicinal herb *Oldenlandia diffusa* from confused species [14]

The herb of *Oldenlandia diffusa* (Willd.) Roxb. is a well known folk-medicine in China as a component of herb tea. Two other species of the same genus, namely, *O. corymbosa* (L.) Lam and *O. tenelliflora* Bl. had been found sold as substitutes in commercial herbal markets. In order to find a quick and easy method to distinguish *O. diffusa* from these similar species of the same genus, the fluorescence microscope was used to investigate the fluorescence emission characteristics of the three tissues as compared with light microscopy images of the same material. Based on the shape of transverse sections of the stem, *O. diffusa* can be easily differentiated from two similar species of the same genus. When seen with the routine light microscope, the microscopic characteristics of the stems from *O. tenelliflora* and *O. corymbosa* were very similar; however the two herbs could easily be identified using the fluorescence of the endodermal cell walls (Fig. 2 and 3). Specifically, the wall of endoderm cells of *O. diffusa* and *O. tenelliflora* emit autofluorescence, while comparable tissue of *O. corymbosa* does not.
**Fig. 2** Transverse sections of the stems as seen under light microscope (×50)

A. *O. diffusa*; B. *O. corymbosa*; C. *O. Tenelliflora*
Fig. 3 Transverse sections of the stems under light microscope and fluorescence microscope (×400)
A. *O. diffusa*; B. *O. corymbosa*; C. *O. tenelliflora*


A-1, B-1, C-1: emission filter through long-pass 397nm; A-2, B-2, C-2: emission filter through long-pass 515 nm
Identification of Chinese Proprietary Medicines

Wu Zi Yan Zong Wan, as a classical Chinese Medicine Formula, is used for replenishing the kidney with vital essence. Both Chinese proprietary medicines and healthcare products prepared according to this formula circulate not only in China, but also in many other countries such as Japan and the United States. The microscopic identification of Wu Zi Yan Zong Wan was carried out together with examination of crude samples of its 5 component herbs to ensure the presence of these constituents in the herbal products (Fig. 4) [24].

[Fig. 4 Identification of Wu Zi Yan Zong Wan]

1. Original plant 2. Crude drugs 3. Key features of microscopic identification

3.2 General Approach to Microscopic Identification of Chinese proprietary medicines

In general, microscopic identification of Chinese proprietary medicines can be classified into two situations. One is dealing with known constituents, as in the above example; the second is when the constituents are unknown and need to be identified. In the latter case, identification is much more difficult, because we must know the microscopic features of virtually all single CHM powders in order to know which one(s) are present. However, apart from the microscope, a variety of other techniques can also be used in identification. For example, some CHMs in the prescriptions, which do not have very specific microscopic features, like Borneol (Borneolum syntheticum), can be identified by microsublimation. Moreover, fragments of powdered crude drugs constituents are usually partly hidden by several other co-existing crude drugs and additives. In such cases, polariscopic identification is suggested to be the first choice. For example, polarized microscopy has been applied to identify the ingredients of Zhibao Sanbian Wan, which is in the form of a large honey pill comprising 38 kinds of powdered crude drugs as a traditional Chinese Medicine commonly used for tonicity in China and Japan [25].
When using microscopy to identify Chinese proprietary medicines, some points should be noted:
(1) Know the form of medication, and be familiar with the constituents of prescription;
(2) Eliminate overlapped interference; and identify specific features;
(3) Master the standard operations;
(4) Strive for accuracy.

4. Use of the Microscope in Quality Evaluation of CHMs

As CHMs are increasingly used worldwide, evaluating the quality and ensuring the correct identify of CHMs has become an internationally critical issue. Confused species or inferior quality of herbal materials can, at the least, degrade the curative effects of herbal medicines or, at the worst, induce poisoning. Microscopic identification is an effective means for identifying CHMs plant material. However, authentication of CHM includes both identifying the crude plant product and evaluating its pharmaceutical quality. Today, quality evaluation mostly depends on complex and expensive instruments operated by professional technicians, and is recorded in terms of active metabolites. If biologically active components can be correlated with microscopic characteristics, then evaluating the quality of CHM becomes much more practical and can become widespread, with many beneficial results.

It is well known that the metabolites—including the pharmacologically active components of CHMs—distributed in plant tissues of microscopic characteristics have a specific, predictable relationship with quality of CHMs. That is to say, analysis of chemicals in plant tissues will assist to evaluate the quality of CHM by quantification of plant tissue which contain the active components. The study on the analysis of chemicals in plant tissues has been reported. The technique of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) has been applied for histochemical analysis in our previous studies. The results demonstrated that the plant tissues have a close correlation with chemical components. For example, the results of in vivo analysis and spatial profiling of chemicals in stem tissue of Sinomenium acutum (Thunb) Rehd. et Wils by MALDI-TOF-MS indicated that the chemicals (+)-menisperine, stepharanine, sinomenine and sinomendine are mainly distributed in xylem while magnoflorine is found in pith [26]. In another of our previous studies, it was found that the content of sinomenine in the plants of large size (stem diameter > 3 cm) was much higher than those of small size (stem diameter < 1 cm) [27]. Both the two previous studies showed that the active chemicals were distributed in the xylem tissue of the stem of S. acutum. Therefore, the microscopic characteristic of the stem tissue of S. acutum most valuable for determination of pharmaceutical quality is the quantity of vessels. Additionally, the alkaloid profiling in plant tissue of the root of Aconitum carmichaeli Debx. as well as crude and processed Strychnos nux-vomica seeds has been successfully analyzed by MALDI-TOF-MS technique [28, 29].

Laser microdissection (LMD), which can separate the different tissues in transverse sections, is proving to be very useful in the authentication of herbal products. For example, LMD combined with cryogenic nuclear magnetic resonance (NMR) and mass spectrometry has been applied to analyse the chemicals in stone cells of Norway spruce (Picea abies) [30]. LMD also has been used to separate the secretory cavities from leaves of Dilatris pillansii Barker. Then cryogenic 1H NMR spectroscopy and HPLC analysis were used to analyze the microdissected samples; this process revealed two novel and one known natural products in secretory tissues [31]. The identification of secondary plant metabolites from a single laser-microdissected population of plant cells combined with sensitive analytical techniques offers a new way to determine the chemical profiles of specific plant cell types with a high degree of precision. In other word, using LMD to localize natural products in specific plant cell populations makes it possible to correlate microscopic characteristics and CHM pharmaceutical quality.

5. Conclusion

In conclusion, microscopic identification is a reliable, efficient and effective method for the authentication of herbal medicines in general and Chinese herbal medicines in particular. However, authentication of CHM includes both identifying the crude plant product and evaluating its pharmaceutical quality. As microscope technology advances, with corresponding advances in analytical chemistry, it is becoming feasible to also evaluate the quality of CHMs from microscopic examination.

References


