The usage of microscopy method for herbal standardizations

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Quality control standardizations of the natural products such as medicinal plants getting more attention in recent years since the commercialization of herbal formulations increased dramatically. A wide range of methods can be applied for standardizations of herbal products. However, advancement in the microscopy technique could help to achieve the goal of standardizations of the herbal products. Moreover, majority of the regulatory procedure and pharmacopoeias propose macroscopic and microscopic evaluation for herbal standardization. Thus, in this chapter we analyzed and discussed the usage of light microscopy technique for herbal standardization. The methods presented in this chapter are illustrated on the model of *Polyalthia longifolia* leaf which is a local herbal plant.

**Keywords** standardizations; microscopy technique; histology and *Polyalthia longifolia* leaf

1. Introduction

The search for therapeutic agents and bioactive compound to fight against emerging and existing diseases is ongoing process and plant especially herbs and edible plants have regularly aimed as part of this search because they reach with various phytochemicals with medicinal properties. Since ancient time man relies on many medicinal plants to treat various diseases. Hence, medicinal plants are being vigorously investigated by world scientist for new lead compounds to developed therapeutics agents. However, the identification and quality control standards of medicinal plants play an important role to exchange of relevant information amongst the scientific research community. Since various types of medicinal plants are investigated throughout the year’s, quality control and uniformity become a major problem in search for therapeutic agents and bioactive compound from plant kingdom. Research activity help to address this problem will be a good task to deal with. In this chapter, we describe and discuss the usage of microscopy method for medicinal plants standardizations such as macroscopic and microscopic technique on the model of *P. longifolia* leaf.

A significant factor, which can add to the consistent quality of medicinal plants, is to have satisfactory standardization. Due to the natural heterogeneity such as varied geographical location where these plants grow, problem of diverse vernacular names these plants known by, the quality of herbal starting materials obtained from wild collections shows great fluctuations. Thus, standardization of herbal products has been extensively promoted during the recent years. Standardization is defined by the American Herbal Products Association as “… the body of information and controls necessary to produced materials of reasonable consistency.” This is achieved through minimizing the inherent variation of natural product composition through quality assurance practices applied to agricultural and manufacturing processes”[1]. For standardization and quality assurance purposes, the following three attributes are must be verified: authenticity, purity and assay [2] as shown in Fig. 1.

Authentication of plants material refers to many parameters such as gross morphological observation, macroscopy and microscopy observation, chemical analysis and DNA fingerprinting. Smillie and Khan [3] reported that the macroscopic observation identification/authentication include traits such as woody/ suffruticose (semi-woody)/herbaceous; leaf shape, size, and morphology (e.g., leaf margins—entire, undulate, dentate, serrate, lobed, pinnatifid); inflorescence characteristics, such as type of inflorescence (e.g., spike, raceme, panicle, cyme, corymb, helicoid cyme, head); floral morphology (e.g., epigynous/perigynous/ hypogynous; stamen number and shape; number of carpels per ovary; number of seeds per carpel); root characteristics, including surface texture, type (corm, bulb, rhizome), and tissue layering (banding patterns). Macroscopic techniques may be used to discriminate between the desired plant species and plant part and morphologically similar, yet distinguishable, species that could occur as potential adulterants. Alternatively, the microscopic approach utilizes techniques such as light microscope microscopy to analyze characteristics such as the presence or absence of hairs (trichomes), oil glands, canals, particular cell types, seed or pollen morphology, and vascular traces [3]. Purity component of medicinal plant standardization relay to evaluation of the presence of adulterants in the plant material which will affect the purity of herbal material or products. The assay component of medicinal plant standardization is concern about chemical and biological profiling which could assess the chemical effects and curative values. The use chemical fingerprint of plant material tends to focus on identification of the stability of the chemical constituents which can used to ensure that the presence of the chemical marker in the newly collected plant material in reproducible manner which can facilitate the manufacturer in controlling the consistency of plant material [4]. Consistency in the biological activity is an essential requirement for the effective use of therapeutic agents. This consistency ensures that the plant material retains its therapeutic parameters throughout the shelf life assigned to the plant material [4].
2. Microscopy technique in authentication

In olden times, authentications of the herbal plants depended on the experienced collector’s knowledge with the plants organoleptic characteristics, such as color, texture and odour. This technique of evaluation by experienced collectors became less reliable, as the sources of medicinal plants broadened and adulteration became more complicated. Development of reliable, easy, cheap, quality and convenient methods for authentication is important for the identification and standardization of medicinal plant, predominantly, toxic plants. Since the advancement in the microscopy technique could help to achieve the goal of standardizations of the herbal products, authentication methods of medicinal plants have been enhanced by the macroscopic identification methods and microscopic identification method. The light microscopic method (LMM) of identification of medicinal plants has been applied for many years and was published by Chinese Pharmacopoeia, as well as in other reference books such as: Chinese Materia Medica, New compendium of Chinese Materia Medica, British Herbal Pharmacopoeia, American Herbal Pharmacopoeia, Japanese Pharmacopoeia, Korea Herbal Pharmacopoeia, and Indian Ayurvedic Pharmacopoeia [5].

Light microscopic method offers several advantages over conventional authentications of the medicinal plants including the effectiveness, simplicity, and low cost, and has been widely adopted as an official method in many international herbal pharmacopoeias as mention earlier. However, there are some limitations of this method such as usage of outdated instruments and equipment, microscopic characters were simply described by words or coupled with pictures drawn by hands, and none of them provide detailed illustrations or photos of both transverse sections and powder characteristics. On the other hand, the advancement in the microscopy technique and the development of digital imaging techniques, much detailed and accurate information about microscopic features can be obtained [6]. Fig. 2 shows the various steps in the authentications of **P. longifolia** leaf by microscopic method. As shown in the Fig. 2 the plant sample will be collected particularly the parts of interest such as leaf, fruit, stem and etc. Subsequently, the plants sample will be observed macroscopically for their morphology, color, texture and odour. Afterward, the plants sample will be subjected to various processes for the preparation of plant tissue for histological observations under the light microscope. An example of the usage of light microscopy technique for herbal standardization on the model of **P. longifolia** leaf which is a local herbal plant will be discussed in following sections.
3. Quality control methods for *P. longifolia* leaf (WHO-Geneva)

The pharmacognostical standardization i.e., quality control methods (WHO-World Health Organization-Geneva) is comprises of various macroscopic and microscopic examination. In this chapter the morphological and microscopic microtome sections of *P. longifolia* leaf will be explained as model.

3.1 Plant collection

*Polyalthia longifolia* leaves were collected from various locations at Universiti Sains Malaysia (USM) Penang, Malaysia and authenticated by botanist from School of Biological Sciences, Universiti Sains Malaysia where the herbarium was deposited. After that the *P. longifolia* leaf was subjected to macroscopic examination with a wide angle dissecting light microscope.

3.2 *P. longifolia* leaf processing for microscopic examination

The preparation of plant tissue for histology often require up to two weeks before samples can be sectioned and examined under light microscopy. However, small plant tissue such as young leaves *P. longifolia* can be processed in short period of time. Principally, there are mainly three processes are involved in tissue preparation namely fixation, dehydration, and embedding.

3.3 Fixation of *P. longifolia* leaf sample

Fixation is done to the tissue/ cellular structure and may require up to two days by standard protocols. The laboratory microwave with integrated vacuum used to prevent crumbling or shattering of sections. Many variations of time,
temperature, and wattage for fixing, dehydrating, and embedding steps were tested without visible improvement. Then it was discovered that a post-fixation treatment of the tissue in 60 to 70% ethanol (EtOH) completely eliminated crumbling during sectioning. The concentration of EtOH was important; post-fixation with 95% EtOH resulted in complete crumbling. In addition, microwave time was also a factor with optimum results at two to four hours.

3.4 Dehydration of P. longifolia leaf sample

Dehydration replaces the water in tissues with a solvent that is miscible with embedding media such as paraffin or resin. Depending on the exact protocol, dehydration often requires five days or more. Solvents that are commonly used to dehydrate plant tissues include EtOH and tertiary butyl alcohol (TBA). Most protocols begin dehydration with 100% EtOH (v/v) and gradually increase the TBA concentration to 100%. Use of this combination in traditional protocols resulted in excellent sections [7], but the procedure is time consuming. In initial trials, dehydration beginning with 25% EtOH/25% TBA followed by gradual conversion to 100% TBA. A gradual change from EtOH to isopropanol, which reportedly works well with leaf and stem tissue [8], also produced sections that shattered. In addition, when the dehydration step was simplified with a gradual conversion from 70% EtOH to 100% isopropanol at 77°C, the sections were smooth and intact. The time at each step was reduced until sections began to shatter. Five minutes was the minimum time necessary at each step for complete dehydration of tissue samples.

3.5 Embedding of P. longifolia leaf sample

Infiltration with embedding medium often requires three to seven more days [7, 9, 10]. The sections were embedded in paraffin. Ribbons were placed on slides with Haupts A solution [10] as an adhesive and floated on 3% formalin at 48°C; the elevated temperature was crucial to prevent wrinkling of sections from the crown core. Sections of this region of the sample, which requires 10 to 20 sections if sectioned completely, are particularly susceptible to wrinkling as well as shattering. As soon as the paraffin ribbon had expanded and sections were wrinkle free (~2 min), they were transferred to a 40°C slide warmer to finish drying. The only difficulty encountered in this step was that because paraffin does not absorb microwave energy, it did not heat very well in the microwave oven. Therefore, to keep the paraffin in a liquid state during embedding, the paraffin had to first be melted in a convection oven at 65°C. Then the container of melted paraffin was placed in a water bath, which was maintained at 65°C in the microwave oven. The dried slides were submerged in xylene for 30 min to dissolve paraffin before sections were triple stained with Safranin, Fast Green, and Orange G (Fisher Scientific, Pittsburgh, PA) as described [10]. A cover-glass was added to slides with Permount adhesive (Fisher Scientific, Pittsburgh, PA). Sections were viewed with a wide angle dissecting light microscope.

4. Results and discussion

4.1 Macroscopic characteristics

P. longifolia is a handsome evergreen tree, forty feet, in height and eleven feet, in girth, with symmetrical pyramidal growth with willowy weeping pendulous branches (Fig. 3a). The macroscopic characteristics of the leaf (Fig. 3b) as follows: Leaves are without petioles and dark green in color. The leaflets are in pair of 3 to 6, oblong or lancet shaped, 4 to 9 inch long and 2.5 inch wide. New leaves with copper tinge appear continuously in groups. The leaf margin is another tool in plant identification. P. longifolia leaf has undulate wave-like margin. P. longifolia has pinnate venation with veins extend from a midrib to the edge. The primary lateral veins were thin and straight. The taste and odour of the leaf were characteristic aromatic and agreeable.

4.2 Microscopic characteristics of transverse section P. longifolia leaf through midrib

Transverse section of the leaf through the midrib showed bowl shaped abaxial parts and straight adaxial side. Both the adaxial and abaxial epidermal layers were single layered thin walled cubical cells. The epidermal cells wide, polygonal, thin walled and the walls were straight or slightly wavy. The lower epidermis was stomatiferous. The lower epidermal cells were smaller as compared to the upper epidermal cells. The epidermal cells followed by four to six layers of angular collenchyma cells on both the sides. In the midrib region, vascular bundle is encircled by a schlerenchymatous ring. Bundle sheath, xylem and phloem are clearly visible (Fig. 4).
4.3 Microscopic characteristics of transverse section *P. longifolia* leaf through lamina

The lamina of the *P. longifolia* leaf is dorsiventral, mesomorphic, amphistomatic, globroscent and even. Both the epidermal cells were squarish to rectangular, cuticle was thin and smooth. Palisade tissues were single layered. They were cylindrical less compact and occupy one third of the thickness of the lamina. Spongy tissues were three-four layered, spherical and less compact. Lateral veins were smaller with few xylem and phloem element (Fig. 5). Cluster of calcium oxalate crystals were found in parenchyma cells of this region.

4.4 Application of microscopic and macroscopic methods in plant identification

Although there has been dramatic increase in interest in the usage of medicinal plants, much of the research to date has been cursed by research conducted using unstandardized plant material. One of the most important issues involved in medicinal plant research study is the quality of the plant material. A research cannot be considered scientifically sound or valid if the material tested was not standardized such that the plant material can be reproduced.\(^\text{18}\) Therefore, microscopic and macroscopic technique will be very helpful to tackle this problem. Macroscopic techniques may be useful to discriminate morphologically similar plant to distinguish between the desired plant species and plant part in the field during the plant sampling. Alternatively, microscopic approach utilizes techniques such as light microscopy to analyze characteristics such as the presence or absence of particular cell types will help to distinguish between the desired plant species and plant part at ultrastructural level. Moreover, microscopic and macroscopic methods can assist the pharmacologist in order to gain a standard botanical based resolution to common identity related questions which concerning about the medicinal plants.

5. Conclusion

Standardization is an important tool for medicinal plant based research in order to ascertain their identity, purity, safety and quality. In order to standardize *P. longifolia* leaf, various macroscopic, microscopic analyses were used in this chapter. To ensure the quality of plant material, the macroscopic and microscopic description of *P. longifolia* leaf is the first step towards establishing it identity and purity. In conclusion, for identification and evaluation of medicinal plants by pharmacognostical studies microscopic method is one of the cheapest, reliable and simplest methods to start with establishing the correct identification of the source material.
Fig. 4 Transverse section of *P. longifolia* leaf midrib view. X: xylem; Ph: phloem; MB: median bundle; MC: mucilage cavity; GT: ground tissue; AdB: accessory adaxial bundle; Ads: adaxial side; Abs: abaxial side; Ta: tanniferous cell; Ep: epidermis; La: lamina;
Acknowledgements
Subramanion L Jothy was supported by MyPhD fellowship from Ministry of Higher Education, Government of Malaysia, Malaysia.

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