Chapter 11
Microscopy in Pharmacognosy

Abstract Microscopy is useful for the study of the internal structure, constitution, and inclusions of plant and animal cells or other objects in detail. It is necessary for the detection of adulterants and contaminants of the herbal preparations and thus provides means for assessing the authenticity and quality of herbal drugs. Size, shape, relative position of different cells and tissues as well as the chemical nature of the cell walls, and the form and nature of cell contents are considered during microscopic analysis of crude drugs. Electron microscope uses electron beam to illuminate a specimen and thus has greater resolving power than a light microscope which uses visible light. Depending on the number of eyepieces or ocular lenses, a microscope may be mono-, bi-, and trinocular, and bright-field, dark-field, phase-contrast, florescence microscope, etc., are light microscopes while transmission, scanning, reflection, scanning transmission, low-voltage electron, etc., are electron microscopes. Botanical microscopic atlas uses the characteristics of botanically authenticated multiple samples that have been compared and cross-checked against other microscopic characterizations for consistency and completeness. Microscopic evaluation of botanical drugs may be of both qualitative and quantitative. Qualitative microscopy includes studies of the transverse sections of leaf, root bark, as well as longitudinal section of root bark under photomicrograph with or without staining. In case of powder microscopy, different staining reagents such as iodine for detection of starch grains and calcium oxalate crystals while phloroglucinol for detection of lignified components are used. Quantitative microscopy of some pharmacognostic parameters like vein-islet number, vein termination number, stomatal number, stomatal index, and palisade ratio are used for identification, purity determination, and evaluation of crude leafy drugs. Drawing of morphological and histological structures of plant and animal organs and various other minute structures (e.g., trichomes, glands, stomata, calcium oxalate crystals) is also used for quantitative microanalysis of admixed or adulterated powdered drugs. Plant sections or powders of the drug are mounted in water or dilute glycerol for light microscopic examination. Color and clearing, bleaching and defatting reagents are used to stain and clear prior to microscopic examinations. Tissues are macerated by using chemicals to disintegrate the middle lamella and isolation of tissues for study. The characteristic microscopic features include trichomes,

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palisade and spongy parenchyma, collenchyma, stomatal frequency, their index, vein-islet, vein termination number, palisade ratio, shape and size, as well as vascular bundles, xylem and phloem cells, inclusions, etc., and their physical constants for leafy drugs while cork cambium, primary cortex, phloem fibers, medullary rays, endodermis, pericycle, vascular bundles, etc., in the transverse and longitudinal sections, and their physical constants stand as characteristic microscopic features of drugs from root, stem, etc. Micrometry and camera lucida drawing to scale of tissues, cells, cellular elements, cell inclusions, and other minute structures are of significant value in the examination of crude drugs for quality assessment in presence of adulterants. With the worldwide increase in popularity and acceptance of herbal medicines, the classical tool like microscopy is urgently needed for the assessment and quality control of plant products such as crude herbal drugs, registered herbal medicinal products, over-the-counter herbal products, or health foods. Modern pharmacopoeias offer new monographs on herbal drugs, including their microscopic characterization.

Keywords Microscopy · Light and electron microscope · Botanical microscopic atlas · Micrometry · Camera lucida drawing

Pharmacognosy is an applied science, and it utilizes many techniques and procedures originated and developed by ancillary sciences. Sometimes such techniques have been modified to better meet the peculiar needs of quantitative microscopy (Hampton Hoch 1948). Microscopy (microscopic examination) is an important technique used in pharmacognosy for the study and identification of crude drugs. According to American Herbal Pharmacopoeia, microscopic characterizations of botanical medicines introduce botanical microscopy to the industry as a low-cost quality assessment tool for the physical examination of botanicals and highlight the value of botanical microscopy as an important physical assessment tool for botanicals (Anonymous 1911). Microscopy provides methods for assessing the authenticity and quality of herbal drugs.

Microscope (also magnifying lens) is an optical instrument that magnifies smaller objects and thus helps to study their details—structure, constitution, and inclusions. It is also used to study the details of the internal structure and contents of large objects, plant parts, and animal tissues. Based on light sources for transmission (visible or electronic beam), microscopes are classified into two broad groups: (i) Light microscope, both simple and compound, uses visible light from sun or electric bulb (Figs. 11.1 and 11.2), and (ii) Electron microscope, which uses an electron beam to illuminate a specimen (Fig. 11.3). An electron microscope has greater resolving power than a light microscope and can reveal the structure of smaller objects because electrons have wavelengths about 100,000 times shorter than visible light photons. Light microscope includes bright-field, dark-field, and phase-contrast and florescence microscope while electron microscope may be

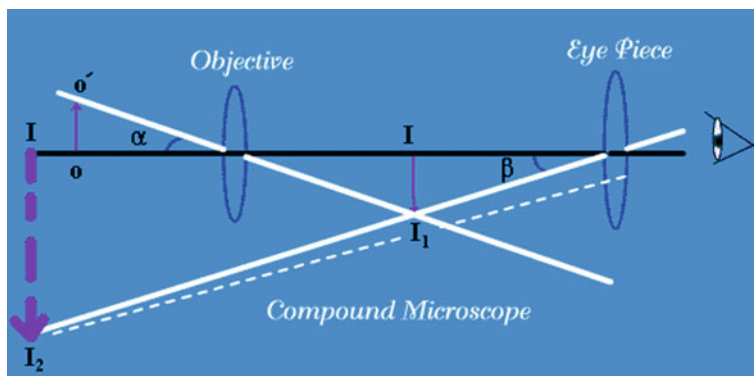

Fig. 11.1 Working principle and magnification of microscope

transmission electron microscope, scanning electron microscope, reflection electron microscope, scanning transmission electron microscope, and low-voltage electron microscope (Fig. 11.3). Microscope may be mono-, bi-, and trinocular depending on the presence of the number of eyepieces or ocular lenses.

11.1 Construction and Working Principles of Compound Microscopes

The simple microscope has limited magnifying (10×) capacity. The compound microscope (common light microscope used in the laboratory) produces high magnification. It is basically made by using two convex lenses of short focal length. They are arranged vertically on a common axis at certain distance from each other. The first of these lenses (lower one), called the objective lens, produces the primary image (I) of the object (O) placed under it. This is an enlarged, real, inverted image of the object. This image then acts as an object for the second lens (upper one), called the ocular or eye lens, which gives a still further enlarged virtual image (I) of the object. This is the image, which is seen by the eye of the observer. Magnification of a specimen is the function of a two-lens system; the ocular lens is found in the eyepiece, and the objective lens is situated in a revolving nosepiece.

Microscope magnification calculation examples: When the ocular lens is 10× and the objective lens is 10×, the field of view is 800 μm. How to calculate the field of view when the ocular lens is 40× and the objective lens is 100×? The magnification at lower power is $10 \times 10 = 100$. The magnification at higher power is $40 \times 100 = 4000$, higher power field of view = $100 \times 800/4000 = 20$ μm.

A compound microscope essentially contains the following components:

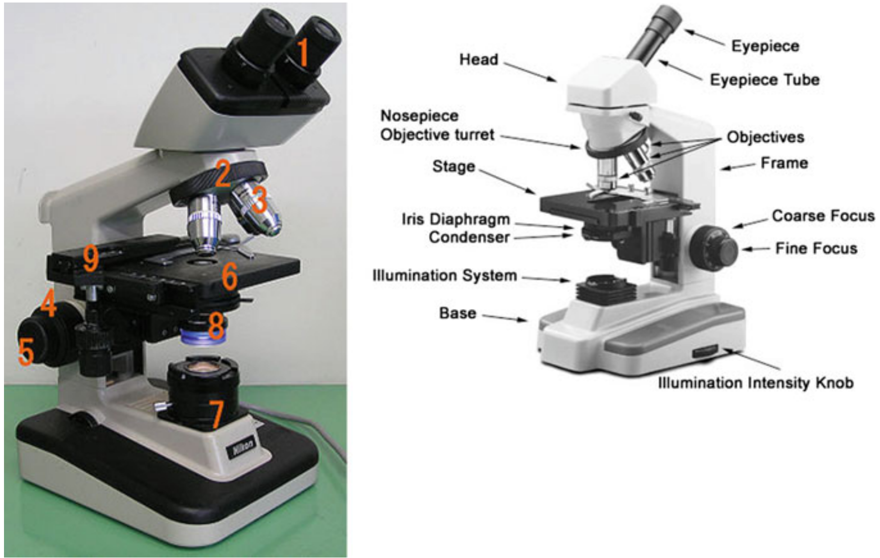

Fig. 11.2 A compound binocular microscope (light). Ocular lens (eyepiece) (*1*), objective turret or revolver or revolving nosepiece (to hold multiple objective lenses) (*2*), objective (*3*), focus wheel to move the stage (*4*—coarse adjustment, *5*—fine adjustment), frame/stage (*6*), light source, a light or a mirror (*7*), diaphragm and condenser lens (*8*), stage and clip (to hold sample) (*9*)

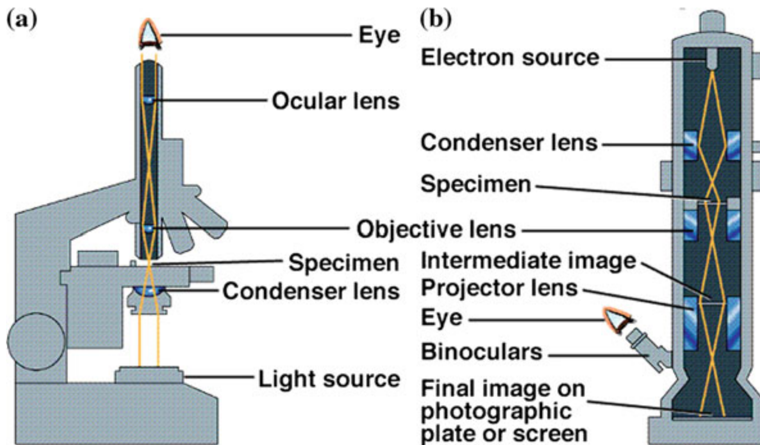

Fig. 11.3 Comparison of **a** compound optical microscope and **b** transmission electron microscope, TEM

A compound microscope essentially consists of the following parts:

(i) **Eyepiece**: The lens closest to the eye is called the eyepiece or ocular. It is the uppermost component part of the microscope through which it holds the eye lens of the microscope.

(ii) **Drawtube**: This is a vertical metallic tube, which houses the eyepiece at its upper end and the nosepiece at the lower end. It moves up and down by two adjustment knobs (coarse and fine).

(iii) **Nosepiece:** This is moveable circular metallic disk fitted at the lower end of the drawtube and holds the objectives.

(iv) **Objective:** The lens closest to the object is called the objective. The objective is a small metallic tube, which holds the objective lens. It screws into the nosepiece.

(v) **Stage:** The stage is a dark colored rectangular or circular metallic support on which the object (mounted on a glass slide) is placed for examination. It is fitted below the nosepiece and is provided with a central hole to allow light to pass through the object. It has two clips to hold the slide with the object in position.

(vi) **Condenser:** It is an optical part on a metallic casing under the stage which condenses light rays to a strong beam to illuminate the object correctly.

(vii) **Diaphragm:** is located on the condenser and controls the amount of light coming through it. Both coarse and fine adjustments are found on the light microscope. The condenser is provided with an adjustable diaphragm, which controls the amount of illuminating light.

(viii) **Light source:** The object is illuminated either by a built-in light source fitted below the condenser, or by an external source of light. In the latter case, the light is reflected to the object by an adjustable plano-concave reflector mirror fitted under the condenser.

(ix) **Stand:** In order to carry the eyepiece, objective, and condenser in strict alignment along the optical axis and to maintain the stage perpendicular to the axis, the microscope is assembled in a strong mechanical frame, called the stand. The stand is provided with a heavy foot, often shapes like a horse shoe, in order to ensure stability of the instrument, and a limb which supports the optical unit, the stage and the adjustable knobs. The limb is attached to the foot by a hinged joint so that the microscope may be set at any desired angle.

Magnification is the ratio of enlargement between the specimen and its image (either printed photograph or the virtual image seen through the eyepiece). To calculate magnification, we multiply the power of each lens through which the light from the specimen passes, indicating that product as GGG×, where GGG is the product. For example, if the light passes through two lenses—an ocular lens 10× and an objective lens 4×, we multiply the 10× ocular value by the value of the objective lens to get the product as $10 \times 4 = 40$, or 40× magnification.

A compound light microscope often contains four objective lenses such as the scanning lens (4×), the low-power lens (10×), the high-power lens (40×), and the oil-immersion lens (100×). With an ocular lens that magnifies 10 times, the total

magnifications possible will be 40× with the scanning lens, 100× with the low-power lens, 400× with the high-power lens, and 1000× with the oil-immersion lens. Most microscopes are parfocal, i.e., the microscope remains in focus when one switches from one objective to the next objective.

Working distance is the distance between the specimen and the magnifying lens. Depth of field is a measure of the amount of a specimen that can be in focus. Magnification and resolution are terms used frequently in the study of cell biology, often without an accurate definition of their meanings. Magnification is a ratio of the enlargement (or reduction) of an image (drawing or photomicrograph), usually expressed as ×1, ×1/2, ×430, ×1000, etc. Resolution is the ability to distinguish between two points. Generally, resolution increases with magnification, although there does come a point of diminishing returns where you increase magnification beyond added resolution gain.

Resolution of a microscope is the ability to see clearly two items as separate objects under the microscope. The resolution is determined in part by the wavelength of the light used for observing. Visible light has a wavelength of about 550 nm, while ultraviolet light has a wavelength of about 400 nm or less. The resolution of a microscope increases as the wavelength decreases, so ultraviolet light allows one to detect objects not seen with visible light. The resolving power of a lens refers to the size of the smallest object that can be seen with that lens. The resolving power is based on the wavelength of the light used and the numerical aperture of the lens. The numerical aperture (NA) refers to the widest cone of light that can enter the lens; the NA is engraved on the side of the objective lens.

If the user is to see objects clearly, sufficient light must enter the objective lens. With modern microscopes, entry to the objective is not a problem for scanning, low-power, and high-power lenses. However, the oil-immersion lens is exceedingly narrow, and most light misses it. Therefore, the object is seen poorly and without resolution. To increase the resolution with the oil-immersion lens, a drop of immersion oil is placed between the lens and the glass slide. Immersion oil has the same light-bending ability (index of refraction) as the glass slide, so it keeps light in a straight line as it passes through the glass slide to the oil and on to the glass of the objective, the oil-immersion lens. With the increased amount of light entering the objective, the resolution of the object increases, and one can observe objects as small as bacteria. Resolution is important in other types of microscopy as well.

In addition to the familiar compound microscope, microbiologists use other types of microscopes for specific purposes. These microscopes permit viewing of objects not otherwise seen with the light microscope. (i) An alternative microscope is the dark-field microscope, which is used to observe live spirochetes, such as those that cause syphilis. This microscope contains a special condenser that scatters light and causes it to reflect off the specimen at an angle. A light object is seen on a dark background. (ii) A second alternative microscope is the phase-contrast microscope. This microscope also contains special condensers that throw light ‘out of phase’ and cause it to pass through the object at different speeds. Live, unstained organisms are seen clearly with this microscope, and internal cell parts such as mitochondria, lysosomes, and the Golgi body can be seen with this instrument.

The fluorescent microscope uses ultraviolet light as its light source. When ultraviolet light hits an object, it excites the electrons of the object, and they give off light in various shades of color. Since ultraviolet light is used, the resolution of the object increases. A laboratory technique called the fluorescent-antibody technique employs fluorescent dyes and antibodies to help identify unknown bacteria.

Electron microscopes work in similar way as normal optical microscopes. Electron microscope uses beam of accelerated electrons (up to 100,000 times shorter than that of visible light photons) as a source of illumination while light microscope uses visible light spectrum for this purpose. Because of this fact, the electron microscope has a higher resolving power than a light microscope and can reveal the structure of nano-objects and even an atom. A transmission electron microscope (TEM) can achieve better than 0.5 Å or 50 pm resolution and magnifications of up to about 10,000,000× whereas most light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000×.

The transmission electron microscope uses electrostatic and electromagnetic lenses to control the electron beam and focus it to form an image. These electron optical lenses are analogous to the glass lenses of an optical light microscope.

The electron microscope uses electrostatic and electromagnetic 'lenses' to control the electron beam and focus it to form an image. These lenses are analogous to, but different from the glass lenses of an optical microscope that forms a magnified image by focusing light on or through the specimen. Electron microscopes are used to observe a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals. Industrially, the electron microscope is often used for quality control and failure analysis. Since the beam of electrons has an exceptionally short wavelength, it strikes most objects in its path and increases the resolution of the microscope significantly. Viruses and some large molecules can be seen with this instrument. The electrons travel in a vacuum to avoid contact with deflecting air molecules, and magnets focus the beam on the object to be viewed. An image is created on a monitor and viewed by the technologist.

Typical magnification of a light microscope, assuming visible range light, is up to 1500× with a theoretical resolution limit of around 0.2 μm or 200 nm. Electron microscopes were developed due to the limitations of light microscopes which are limited by the physics of light to 500× or 1000× magnification and a resolution of 0.2 micrometers. In the early 1930s, this theoretical limit had been reached, and there was a scientific desire to see the fine details of the interior structures of organic cells. This required 10,000× plus magnification which was just not possible using light microscopes.

11.2 Morphological and Anatomical Examination of Crude Drugs

Botanical identity of crude drugs can be achieved by macro- and microscopic examinations using hand lens, simple or compound microscope. Voucher specimens are reliable reference sources. Outbreaks of diseases among plants may result in changes to the physical appearance of the plant and lead to incorrect identification. At times, an incorrect botanical quality with respect to the labeling can be a problem. For example, in the 1990s, a South American product labeled as 'Paraguay Tea' was associated with an outbreak of anticholinergic poisoning in New York. Subsequent chemical analysis revealed the presence of a class of constituents that was different from the metabolites normally found in the plant from which Paraguay tea is made.

Morphological features or macroscopic characteristics of various plant organs like leaves, flowers, seeds, fruits, barks, root, rhizome, tubers, etc., are considered in the identification of crude drugs. These are organized drugs (with cellular structure), and there are unorganized (with acellular structures). Morphological characteristics of a drug may be divided into four headings viz. (i) shape and size, (ii) color and external markings, (iii) fracture and internal color, and (iv) odor and taste. Morphological features for the identification of leaves include their size, shape, type (simple, compound), sessile or petiolate, glabrous or hairy, if hairy, types of hairs (trichomes) present, type of venation, margin, apex, and base. Flowers can be identified partly by the nature of the inflorescence, partly by the nature of the various floral parts (calyx, corolla, filament, anther, stigma), their external features, and the structure of the perianth (sympetalous, actinomorphic, zygomorphic), and partly by the carpels (monocarpous, apocarpous, syncarpous). Seeds are usually identified by their size, shape, and external color, as well as by the form, arrangement, relative development of the embryo and its parts, and various markings (wrinkles, hilum, and raphe) and outgrowths (hairs, warts) on the surface. Features of fruits for their identification include type (simple, compound, aggregate, capsule, legume, berry), condition (dry, fresh, succulent, ripe, green), shape, size, and external markings (scars, rough, wrinkled). Barks are identified by their form (flat, curved, quailed), size (small pieces, big pieces), external markings (fissures, furrows, wrinkles, corrugations, lenticels), and presence or absence of cork, epiphytes (lichens, moss, etc.). Common features considered in the identification of roots, rhizomes, and tubers include shape (cylindrical, conical, fusiform, napiform), size (length and diameter), presence or absence of aerial parts, condition (woody, fleshy, succulent), and external markings.

In entire form, the drug is kept without any spectacular change in the natural gross morphology. This form is common in cases of seeds, flowers, fruits, leaves, and some roots and rhizomes; or they may be cut, broken, or sliced, as in woods, barks, many roots, and a few rhizomes. They may be more or less matted together, as in Chondrus and in baled leaves; they may be pressed together by hydraulic pressure giving the so-called pressed drugs, or they may be powdered and then

molded into forms, as rhubarb fingers. Drugs derived from underground parts of the plant, such as rhizomes, roots, bulbs, corms, and tubers, may be either (i) entire, (ii) in longitudinal slices, (iii) in oblique or transverse slices, (iv) cut in small cubical pieces, or (v) broken into pieces. Sometimes the periderm is removed, as in roots (Russian Licorice), rhizomes (Ginger), and barks (Sassafras). Mexican Sarsaparilla may come to the market in neat cylindrical rolls in which a mass of the bundled roots is tightly wrapped by many coils of long roots, or the roots may be in tightly packed bales, or it may be cut into short pieces, or be coarsely ground or finely powdered: Each of these forms presents a very different appearance, yet all are the same drug.

Barks are tissues in a woody stem outside the inner fascicular cambium, e.g., Cinnamon, Cinchona, Quillaia, Ashoka, and Kurchi. Underground drugs are often swollen due to storage of carbohydrates and other chemicals, e.g., roots (Podophyllum, Liquorice, Jatamansi, Rauwolfia), rhizomes, and stolons have buds, scale leaves, and scars (Ginger, Turmeric, Dioscorea). Leaves arise from a node on a stem and leafy drugs like Senna, Tulsi, Vasaka, Digitalis, etc., and leaves can be easily identified on the basis of their shape, margin, base, apex, and venation. Flowers possess different shapes, size, and color, e.g., Saffron, Banafsha, Pyrethrum. Fruits arise from the ovary and contain seeds, e.g., Cardamom, Colocynth, Almond, Vidang, Bahera, Amla, and Bael. Seeds are developed from the ovules in carpels of the flowers and characterized by the hilum, micropyle, and sometimes raphe. The seed drugs are Ispaghula, Linseed, Nux-vomica, Psoralia. Herbs are the whole aerial part, and it is sometimes used as a drug, e.g., Brahmi (*Bacopa monnieri*), Chirata (*Swertia chirata*), Kalmegh (*Andrographis paniculata*), Pudina (*Mentha arvensis*), Shankhpushpi (*Convolvulus pluricaulis*). The shape of a drug may be cylindrical (Sarsaparilla), subcylindrical (Podophyllum), conical (Aconite); fusiform, ovoid or pyriform (Jalap), and terete (tapering gradually) or disk-shaped (Nux-vomica). The drug may be simple, branched, curved, or twisted. The length, breadth, and diameter are measured in millimeters or centimeters. In case of conical drugs, the size of both parts is mentioned.

The parts may be simple or branched and are frequently curved and twisted. In the case of rhizomes, the direction of growth is often considered. This is usually horizontal but may be oblique and in a few cases is vertical. The direction may be roughly determined by the attachment of the roots and stem bases. Sizes are given as to length and diameter and in the most convenient terms, either millimeters (mm) or centimeters (cm). In cases where the shape is conical, the diameter of both wide and narrow parts may be of importance. External markings are classified as furrows, alternating ridges and valleys formed due to shrinkage of internal parts after drying; wrinkles, delicate furrows; annulations, transverse ring-like markings; fissures, splits extending into tissues; nodules, rounded outgrowth on the surface; projections of root, stem bases and buds; scars of leaf, stem-base, root, bud, bud-scale, etc. The fractures may be complete, incomplete, short, fibrous, splintery (breaking irregularly), brittle (easily broken), tough, and weak.

Anatomical or microscopic examination is an important technique, and all modern optical microscopes are designed for viewing samples by transmitted light

and magnify smaller objects and thus help to study their details—structure, constitution, and inclusions of plant parts and animal tissues. Microscopic evaluation is indispensable in the initial identification of herbs, as well as in identifying small fragments of crude or powdered herbs, and detection of foreign matter and adulterants. A primary visual evaluation, which seldom needs more than a simple magnifying lens, can be used to ensure that the plant is of the required species and that the right part of the plant is being used. At other times, microscopic analysis is needed to determine the correct species and/or that the correct part of the species is present. For instance, pollen morphology may be used in the case of flowers to identify the species, and the presence of certain microscopic structures such as leaf stomata can be used to identify the plant part used. Although this may seem obvious, it is of prime importance, especially when different parts of the same plant are to be used for different treatments. Stinging nettle (*Urtica urens*) is a classic example where the aerial parts are used to treat rheumatism, while the roots are applied for benign prostate hyperplasia.

A number of criteria need to be met for the development of microscopic characterizations relevant to botanical identification such as (i) the samples used for the characterizations must be accurately identified by a botanist; (ii) a variety of samples must be used and compared to ensure that the characterization encompasses the natural intraspecies variations that can occur, and (iii) the samples must be representative of the commercial material available in trade. The development of 'Botanical Microscopic Atlas' is to be based on the characteristics of multiple samples that are botanically authenticated, compared against botanical samples in professional herbaria, and cross-checked against other microscopic characterizations for consistency and completeness.

Each microscopic characterization is to be listed primarily according to the Latin botanical binomial nomenclature, including the botanical authority. The botanical nomenclature is then followed by the common name according to Herbs of Commerce (McGuffin et al. 2000), the corresponding pharmaceutical name, and the plant family, which in some cases is diagnostically valuable.

In addition to nomenclature, each microscopic characterization includes four parts: (i) a brief introductory paragraph on the primary medicinal use of the botanical with specific information on potential adulterants of which the microscopist should be aware; (ii) a detailed text description of the microscopic characterization of the plant part in its relatively whole form, along with a listing of the primary tissues found in the same material when it is powdered; (iii) illustrations of the primary tissues that are most prominent and diagnostically relevant to the microscopist; and (iv) photographic images of the primary structures and tissues. The illustrations allow key elements to be highlighted, and the images provide a view of what is actually seen by the microscopist.

11.3 Physical Constants: Techniques and Microscopic Measurement

The characteristic microscopic features include trichomes, palisade and spongy parenchyma, collenchyma, stomatal frequency, their index, shape and size, as well as vascular bundles, xylem and phloem cells, inclusions, etc., and their physical constants for leafy drugs while cork cambium, primary cortex, phloem fibers, medullary rays, endodermis, pericycle, vascular bundles, etc., in the transverse and longitudinal sections, and their physical constants stand as characteristic microscopic features of drugs from root, stem, etc. In powder materials, presence of cortex cells, sieve tubes, calcium oxalate crystals, lignified fibers, etc., are considered characteristic microscopic features. They are considered for both qualitative (study of thin sections or powder of leaf, stem or root, etc.) and quantitative (determination of stomatal number, stomatal index, vein-islet, vein termination number, palisade ratio, etc.) microscopy.

Microscopic examination of section and powder drugs aided by stains helps in distinguishing anatomy of adulterants. Microscopic examination of epidermal trichomes and calcium oxalate crystals is valuable in powdered drugs. In the powdered drugs, the cells are mostly broken, except lignified cells, and the cell contents like starch, calcium oxalate crystals, aleurone, etc., are scattered in the powder. Some fragments are specific for each powder which may consist of parts of cells or groups of cells. Plant parts are made up of specific arranged tissues, spores (Lycopodium), or hairs (Lupulin).

Histological characters are studied from very thin transverse or longitudinal sections properly mounted in suitable stains, reagents, or mounting media. The size, shape, and relative positions of the different cells and tissues, chemical nature of the cell walls and of the cell contents are determined. The basic arrangement of tissues in each drug is fairly constant. Fibers, sclereids, tracheids, vessels, and cork are least affected by drying. Starch, calcium oxalate, epidermal trichomes, and lignin are examined carefully.

Microscope is also used for a quantitative evaluation of drugs and adulterated powders. This is done by counting a specific histological feature such as stomatal index, vein-islets, and vein termination numbers, palisade ratio, etc. These features are compared with the standard samples. Palisade ratio: The average number of palisade cells beneath each epidermal cell is called as palisade ratio. It is determined from powdered drugs with the help of camera lucida.

Importance of the microscope as a tool for the study and analysis of crude drugs became obvious when Jacob Schleiden successfully utilized microscope in 1857 to distinguish various types of Sarsaparilla (*Smilax officinalis* and six other species of *Smilax* of Smilacaceae, native to South America, Jamaica, the Caribbean, Mexico, Honduras, and the West Indies) roots by means of their endodermal cells. Schacht also showed its value in 1953 in the examination of textile fibers. At present, it is one of the most commonly used optical instruments in the study of crude plant drugs.

The microscopic evaluation of drugs is done with the aid of microscopes and utilizes various microscopic characters of the drugs such as trichomes, calcium oxalate crystals, starch grains, pollen grains, etc., and their histological features such as types and arrangements of various cells and tissues. This method of evaluation is indispensable in the evaluation of powdered drugs, as they possess very few macroscopic characters other than color, odor, and taste. Microscope is also essential for determining some important physical constants like stomatal number, stomatal index, palisade ratio, vein-islet number, vein termination number, etc., of leaf drugs. This type of microscopic determinations is otherwise known as quantitative microscopy.

Microscope is useful in both the qualitative and quantitative study and analysis of crude drugs. For qualitative microscopy, transverse sections of leaf and transverse and longitudinal sections of stem and root including their barks are studied under photomicrograph. Leaf microscopy involves the study of external and internal structure and characteristics of lamina (isobilateral or dorsiventral). Color and presence of oil glands in the lamina are also taken into consideration for study. For internal structure, leaf color and pigments are removed by using suitable reagents (e.g., chloral hydrate solution), freehand thin sections are taken by inserting a suitable leaf segments in a potato cube, then mounted on a glass slide in glycerin without or with stains (e.g., methyl orange and phloroglucinol-HCl) as per standard procedures (Wallis 1985; Kokate 2005; Ali 2008; Pandya et al. 2010). Different identifying characters of epidermis, epidermal outgrowth (trichome) and modification (stoma), mesophyll tissue, palisade and spongy parenchyma ratio, vascular bundle (xylem, phloem, cambium and bundle sheath), etc., are then studied for quantification under microscope fitted to camera lucida. For the internal structure of bark, it is softened by boiling in water for few minutes in a test tube containing sufficient water and was boiled for few minutes, and then the softened bark is sliced into fine sections transversally and longitudinally. The stained and unstained sections may be observed under microscope for the identifying characters (Khandelwal 2007; Gupta et al. 2008).

For powder microscopy, a little quantity of the dried powdered material is to be taken onto a microscopic slide, stained by using a little quantity (1–2 drops) of different staining reagents (such as iodine for detection of starch grains, calcium oxalate crystal, and phloroglucinol-HCl solution for detection of lignified components), covered with a cover slip and then studied under microscope the characteristic structures after mounting the preparation in glycerol. The presence of starch grain and calcium oxalate crystal was detected by the formation of blue color on addition of 2–3 drops of 0.01 M iodine solution (Thitikornpong et al. 2011)

Minute morphological structures (trichomes, glands, stomata, calcium oxalate crystals, etc.) of plant organs and their dimensions are very conveniently studied and measured under the microscope. These structures and their sizes are often very useful in the identification of crude plant drugs. Histological structures, which are most frequently used to identify most natural crude drugs and to detect adulterants in them, can only be studied under the microscope in thin sections and in powders. The microscope is indispensable in the identification of powdered drugs and

detection of adulterants in them as they possess very few macroscopic characters other than color, odor, and taste.

Qualitative characters like types of stomata found in different leafy drugs would be the diagnostic characters of different drugs, e.g., (i) paracytic or rubiaceous or parallel-celled stomata in leaf of coca, senna; (ii) diacytic or caryophyllaceous or cross-celled stomata in peppermint, vasaka; (iii) anisocytic or cruciferous or unequal-celled stomata in Belladonna, Datura; and (iv) anomocytic or ranunculaceous or irregular-celled stomata in Digitalis, Lobelia.

Trichome is an elongated tubular outgrowth of an epidermal cell and its function may be protective and also it secretes essential oil and absorbs water. Different types of trichomes are associated with different crude drugs, e.g., (a) covering or non-glandular trichomes [(i) lignified trichomes, (ii) short, sharp pointed, curved, (iii) large, conical, strongly shrunken, (iv) short, conical, warty] in Nux-vomica, Strophanthus, Cannabis, Lobelia, Senna; (b) covering trichomes (multicellular T-shaped trichomes) in Artemisia, Pyrethrum; (c) covering unbranched trichomes [(i) bi-cellular, conical, (ii) three-celled long, (iii) four- to five-celled long)] in Datura, Stramonium, Belladonna; (d) glandular trichomes [(i) unicellular, (ii) multicellular)] in Vasaka, *Digitalis pupurea*, *D. thapsi*, *Cannabis sativa*.

Types or shapes of cell inclusions or ergastic substances like calcium oxalate crystals as seen under the light microscope may often be the distinguishing feature of many crude drugs, e.g., (i) microphenoidal in Belladonna, (ii) prism in Hyoscymus and senna, (iii) raphides in Squill, Rauwolfia, and Cinnamon, and (iv) rosetts in senna, Rhubarb, and acicular crystals in Squill, Ipecacauanha.

Physical constants (leaf constants) like (i) **Palisade ratio**: average number of palisade cell beneath each epidermal cell, it can be determined with powdered drugs; (ii) **vein-islet number**: number of vein-islets per sq. mm of the leaf surface midway between midrib and margin; (iii) **vein termination number**: number of vein-islets termination per sq. mm of the leaf surface midway between midrib and margin; (iv) **stomatal number**: average number of stomata per sq. mm of epidermis of the leaf; and (v) **stomatal index**: percentage which the number of stomata forms to the total number of epidermal cells each stoma being counted as one cell. These are conveniently determined by quantitative microscopy only. These are valuable parameters in the identification and purity determination of crude plant drugs, particularly leaf drugs. Drawing of internal structures of plant and animal organs and various other minute structures to scale and in their exact natural shape and arrangement is done under the microscope. It is also used for quantitative microanalysis of admixed or adulterated powdered drugs.

Palisade ratio represents the average number of palisade cells beneath one epidermal cell, using four continuous epidermal cells for the count. It is determined from powdered drugs with the help of camera lucida. Plisade ratio of *Atropa belladonna*—05–07; *Adhatoda zeylanica*—5.5–6.5; *Cassia angustifolia*—5.5–10 upper, 4.0–7.4 lower (senna); *Digitalis lanata*—2.5–6.5, etc.

For the determination of palisade ratio, a piece of the leaf boiled in chloral hydrate is placed on a slide under microscope. Camera lucida and drawing board are arranged and the outline of four cells of the epidermis is to be traced using 4 mm

objective. Then, palisade layer is focused down, and sufficient cells for covering the tracing of the epidermal cells are traced off. The outline of those palisade cells which are intersected by the epidermal walls is completed. The palisade cells under the four epidermal cells (including cells which are more than half and excluding cells which are less than half within the area of epidermal cells) are counted. The determination for five groups of four epidermal cells from different part of the leaf is to be repeated. The average number of cells beneath epidermal cells calculated is known as palisade ratio.

Stomatal number is calculated as the average number of stomata per square mm of the epidermis. Stomatal number of *Atropa belladonna* were: 07–10 (upper epidermis), 77–115 (lower epidermis); *Datura metel* were: 147–160 (upper epidermis), 200–209 (lower epidermis); and *Ocimum sanctum* were: 64–72 (upperdermis), 175–250 (lower epidermis).

Stomatal index (SI) is the percentage proportion of the number of stomata to the number of total epidermal cells. For determination of stomatal index (S.I.), a piece of leaf is to be cleaned, and the upper and lower epidermis is to be peeled out separately by means of forceps, put on slide and mounted in glycerin (10%) and studied under microscope. Camera lucida is attached, and drawing board is placed for drawing the cells. A square of 1 mm by means of stage micrometer may be drawn on it. The number of stomata and the number of epidermal cells in each field or focus may be counted and averaged, and the stomatal index may be calculated by using the appropriate formula separately for upper and lower surface.

Stomatal index (S.I.) can be calculated by using the following formula:

$$\text{S.I.} = S/S + E \times 100$$

where S = number of stomata per unit area and E = number of epidermal cells in the same unit area.

S.I. of *Atropa belladonna* is 20.2–23.0. Stomatal number varies with the age of a plant but the S.I. for a given plant species generally remains constant throughout the age.

Vein-islet number is the number of vein-islets per sq. mm of leaf surface (photosynthetic tissue) encircled by the ultimate divisions of the conducting strands. Vein-islet number is calculated from four contiguous sq. mm in the central part of the lamina, midway between the midrib and the margin. The ranges of vein-islet in different drug plants vary considerably, e.g., *Andrographis paniculata*—9–12; *Bacopa monniera*—6–13; *Cannabis sativa*—18.24; *Digitalis purpurea*—2.5–3.0; *Eucalyptus globules*—8–13.5; *Cassia senna*—26; *C. angustifolia*—21; *Erythroxylum coca*—11, *E. iruxiuense*—20. Vein termination number is the number of veinlet termination per sq. mm of the leaf surface between midrib and margin. A vein termination is the ultimate free termination of a veinlet or branch of a veinlet. By this character, different coca leaves and senna leaflets are differentiated.

Determination of vein-islet and vein termination number is also considered in quantitative microscopy for herbal drug authentication. Vein-islet is the minute area

of photosynthetic tissue encircled by the ultimate division of the conducting strands. Vein termination number is the number of veinlet terminations per mm of leaf surface. For determination, a piece of the leaf is cleared by boiling in chloral hydrate solution, and camera lucida and drawing board are arranged, and 1 mm line is drawn with help of stage micrometer. A square is to be constructed on this line in the center of the field. The slide is to be placed on the stage. The veins included within the square are to be traced off, completing the outline of those islets which overlap two adjustment sides of the square. The average number of vein-islet from the four adjoining square, to get the value for one square mm, is calculated (Srinivasa et al. 2008). The number of veinlet termination present within the square is counted, and the average number of veinlet termination number from the four adjoining square to get the value for 1 square mm is found known as vein termination number.

Lycopodium Spore Method

Lycopodium is a Pteridophyte plant that has small spore-carrying cones. The number of spores per mg of Lycopodium powder was determined by (a) direct counting and (b) calculation based on specific gravity and dimensions of the spores which gave values in good agreement. As a quantitative microscopic method, Lycopodium spore method is considered for the study of powdered drugs having well-defined particles like pollens, starch grains, single-layered tissues, or some other types of uniformly thick particles. This is an important method employed, especially in identification of *Caryophyllus aromaticus* when chemical and physical methods are inapplicable as accurate measures of quality. On average, uniform size ($\sim$ 25 um) 94,000 spores per mg of powdered Lycopodium are present. By using Lycopodium spore method, the percentage purity of authentic powdered drugs such as ginger (*Zingiber officinale*) can be calculated by using the following equation as:

$$\text{Percentage purity of drug} = (N \times W \times 94{,}000/S \times M \times P) \times 100;$$

where

N number of characteristic structures (e.g., starch grains),
W weight in mg of Lycopodium taken,
S number of Lycopodium spores in the same 25 fields,
M weight in mg of sample calculated on the basis of dried sample at 105 °C, and
P 2,86,000 in case of ginger starch grain powder.

By employing Lycopodium spore method, the number of pollen grains in Pyrethrum powder (1000–2000/mg) and starch granules in wheat powder (400 granules/mg) has been determined. Lycopodium spore method is also useful for the determination of size of a particular type of particle in powders such as epidermal fragments of leaves, single layer of sclerenchyma, or isolated fibers (e.g., epidermal area of Indian senna stalk, sclerenchyma layer in linseed, fibers in the Cinnamon bark) can be measured. The procedure is almost the same as used for counting of particles. The particle size is traced with the help of camera lucida, and the spores

are counted. The tracings are cut out and weighed and their area calculated by weighing a sheet of known area of the paper used. This area divided by the magnification used gives the actual area of the particles in a certain weight of the powdered drug, which is calculated from the number of spores counted and the weight of spores and powder in the suspension. Drawing of internal structures of plant and animal organs and various other minute structures to scale and in their exact natural shape and arrangement is done under the microscope. It is also used for quantitative microanalysis of admixed or adulterated powdered drugs.

11.4 Microscopic Authentication and Quality Assessment of Herbal Drugs

Macroscopic examination can easily be employed to determine the presence of foreign matter, although microscopy is indispensable in certain special cases (e.g., starch deliberately added to dilute the plant material). Determination of foreign matter ensures that the stated herbal drugs are made from the specific part of the plant and are devoid of other parts of the same plant or other plants. They should be entirely free from molds or insects, including excreta and visible contaminant such as sand and stones, poisonous and harmful foreign matter and chemical residues. Animal matters such as insects and invisible microbial contaminants, which can produce toxins, are also among the potential contaminants of herbal medicines. Furthermore, when foreign matter consists, for example, of a chemical residue, TLC is often needed to detect the contaminants.

Standardization of herbal drugs is necessary for the establishment of their identity, purity, safety, and quality. Microscopic method of standardization is one of the cheapest and simplest methods to start with establishing the correct identification of the source material (Singh et al. 2010) and evaluation of purity of drugs (Kumar et al. 2011a, b). Microscopic parameters are distinctive enough to identify and determine the authenticity of herbal drugs and may be included as microscopic standards in the text of Herbal Pharmacopeia. Upton et al. (2011) by putting emphasis on the importance of authoritative microscopic descriptions of the commercially used medicinal plant species for correct identification, provided an atlas containing detailed text and graphic descriptions of more than 140 medicinal plant species and their adulterants. All these may definitely fulfill the needs of the herbal products industry, regulatory agencies, and academic researchers.

Internal structures or histological or anatomical characters (e.g., microscopic structures and measurements) of crude drugs are very valuable in the confirmation of their identities. Drugs which look very similar in their gross morphology differ sufficiently in their histology—thus making it possible to differentiate between them and to identify them correctly. Microscopic structures are most valuable, especially in the identification of powdered drugs as their identification is largely based on the form, the presence, or absence of certain cell type and cell inclusions. The structures

of histological elements as well as the size of individual elements vary in different plants. Measurements of these elements or structures (fibers, stone cell, vessels, trichomes, starch grains, oxalate crystals, etc.) are often of considerable importance in the identification of the source of the drugs.

Microscopical studies involving the quantitative determination of stomatal number, stomatal index value and palisade ratio, vein-islet and vein termination value determination are important in the evaluation of crude drugs. The information obtained from preliminary phytochemical screening will be useful in finding out the quality of the drug and, in addition to that, microscopic as well as morphological studies will provide reliable information for detecting adulteration. To ensure identification of the crude drugs, microscopic analysis should be supplemented with physicochemical analysis of the plant material.

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