Microscopic Evaluation of Herbs and Processed herbs

Microscopic evaluation is a definite method which helps in the identification of crude herbs/ substitutes/ adulterants in addition to macroscopic evaluation. In a group of herbs containing the same chemical compound like hyoscyamine in Belladonna, Hyoscyamus and Stramonium, microscopic character like type of crystals gives the correct identity of the herb. The microscopic features of Senna and Digitals as leaves are the same. But the isobilateral leaf structure, unicellular, warty trichomes, presence of crystals differentiates Senna from Digitalis which has dorsiventral leaf structure, multicellular collapsed trichomes and absence of crystals.

The evaluation includes: Microscopic sections of the herb; Powder study of processed herbs, Quantitative microscopy of herbs, Quantitative microanalysis and Microchemistry of herbs and processed herbs. Any additional useful information for preparation or analysis should be included in the text procedure for individual plant materials for example leaf constants: stomatal number and stomatal index, vein-islet and veinlet termination number and palisade ratio.

Broad guidance

a) Microscopic sections of the herb

In a particular morphological group of herbs, the microscopic/histological characters are mostly similar with a few specific differences. These specific differences form the basis for identification of the crude herb. Sections of the herbs are studied for the arrangement of general tissues and specialized tissues which are specific for the particular herb and are compared with standard literature to identify the herb. Any tissue other than the tissues of the authentic herb indicates adulteration. Prepare two different types of microscopic sections of herbs: transverse sections of leaf, stem, root and bark; longitudinal sections of stem and root including their barks **(Refer Appendix I).**

b) Powder Study of Processed herbs

The study of powders are gaining importance in Pharmacopoeias as it is simple, quick and needs very little of the herb to find tissues of diagnostic value. Prepare the powders as described in **Appendix II.**

Powder study includes: Organoleptic evaluation, Florescence characteristics and Microscopic features.

Organoleptic evaluation

Observe colour, taste, odour and form of the processed herb.

Colour: green (leaves & aerial part); brown (barks & underground organs); specific color (specific herb)
Odour: Do not inhale the powder direct. Rub a little on the palm remove the powder from the palm and smell. Characteristicodour may give identification, specially volatile oil containing drugs
Taste: Put 1-2 drops of water in a small watch glass. Dip a needle in it and dip again in the powder. Mix well with the needle. Dip the little finger in the water extract of the powder and taste. It is necessary to wash the mouth with water before and after tasting the powder, may give an idea about alkaloid containing herbs andbitters.
Form: Feel the powder between thumb and index finger. Describe as smooth, gritty particles and mucilaginous.
Caution: Do not taste unknown herbs.

Florescence characteristics

Observe the Florescence characteristics as described in Appendix III.

Microscopic features

Preparation of slides

Add 5 ml of chloral hydrate solution and 2-3 drops of hydrochloric acid to 200 mg of processed herb in a small test tube and place the test tube in a beaker and heat it on a water bath for 15 minutes or till it is transparent. Cool and allow the solution to settle or centrifuge the solution. Decant or filter or pipette out the liquid into another test tube. Keep it aside to perform tests. Use the residue after diluting with chloral hydrate, and spread on a slide so as to give not more than 10-12 elements in a field. Take 1-2 drops on a slide, add a drop of phloroglucinol and allow it to evaporate followed by a drop of concentrated hydrochloric acid. To avoid air bubbles while placing the cover glass, hold the slide in the left hand and the cover glass with the right hand. Tilt the slide slightly to the left, while placing the edge of the cover glass, to form a line of the liquid along the edge and slowly drop the cover glass. Remove the excess liquid from the slide with blotting paper without applying pressure.

Prepare at least 3-4 slides for powder study. All the tissues may not be present in one slide. The slide should not contain more than 10-12 elements. Dilute if necessary.

Study of the prepared slides

In powders, microscopic feature are scattered as single cells, tissues and fragments of cells. They are seen in surface view (S.V); transverse view (T.V) and longitudinal view, (L.V). Hence a thorough knowledge of tissues in general is an important requirement before the study. This will help in identification of morphological part, based on starch, trichomes, crystals (Table 1), thereby confirm the identification of herb with reference to specific characters followed by micro-chemical/chemical tests and study the slide under low power (10X10 magnification) from left to right and right to left. List out the general and specific (diagnostic) characters.

Table 1: Identification based on trichomes, starch grains and crystals for 10 common herbs

Drug	Trichomes	Starch	Crystals	Specific tissues
Senna	+	-	+	paracytic stomata, warty, unicellular, conical trichomes, cluster crystals, crystal sheath
Digitalis	+	-	-	stomata, typical collapsed trichomes
Cinnamon	-	+	+	typical sclereids, fibres, microcrystals
Cinchona	-	+	+	large fibres, idioblasts of micro-crystals
Ipecac	-	+	+	raphides, tracheids, cork
Liquorice	-	+	+	numerous starch, crystal sheath, large vessels cork
Fennel	-	-	+	Small rosette crystal in endosperm, reticulate, parenchyma, vittae
Nux-vomica	+	-	-	typical lignified trichomes with sclereid bases, endosperm thick cellulosic wall
Clove	-	-	+	Pollen, numerous cluster crystals, typical oil glands
Quassia	-	+	+	Few starch, fibres, large vessels

c) Quantitative microscopy of herbs

A quantitative microscopy of herbs is done by counting a specific histological features, leaf constants: stomatal number and stomatal index, vein-islet and veinlet termination number and palisade ratio. These features are compared with the standard samples.

Stomatal number and Stomatal index

Stomatal number is the average number of stomata per square mm of the epidermal surface of the leaf. This is also known as stomatal frequency, is variable and depends on certain factors like age, growth and climatic zones, but the ratio of the stomatal number of the upper surface to the lower surface is constant in a species. It is determined by counting the stomata in a square mm. Determine *Stomatal number* for both upper and lower epidermis. These may also be determined on larger pieces of epidermal tissue in powders.
Stomatal index (S.I) is the percentage of epidermal cells modified into stomata. It is fairly constant and is independent of the factors like the age, growth and climatic zones. The stomata and the epidermal cells in a unit area are counted by microscopic methods. Determine the Stomatal indices for both upper and lower epidermis.

Palisade ratio
Palisade cells bear a definite relationship to the epidermal cells, a diagnostic feature in the identification of leaves. It is defined as the number of palisade cells under each epidermal cell. This is determined by tracing a group of four or five epidermal cells and calculating the average number of palisade cells for one epidermal cell. A total of 100 epidermal cells are taken for calculations, 4 epidermal cells of 25 readings or 5 epidermal cells of 20 readings to get the count of 100 cells. This may be carried out on leaf pieces. Determine upper epidermis for dorsiventral leaf; upper and lower surfaces in isobilateral leaves. Use either 5 x40 or 10x40 magnification depending on the cell size.

Vein-islet number is defined as the number of vein-islets per sq mm of the leaf surface, midway between the midrib and the margin. The leaf sample, after soaking in water is treated successively with sodium hypochlorite and10% hydrochloric acid to remove Ca oxalate and finally chloral hydrate. The camera lucida is set- up and by means of a stage micrometer the paper is divided into squares of 1 sq mm. In the cleared preparation veins are traced in four continuous squares, in a square of 2 mm×2 mm. Each vein-islet is numbered during counting. The range and average is determined in 10 sets of 2 mm×2 mm area. Observe under 10X (eye piece) and 4X (objective).

Veinlet termination number is defined as the number of veinlet terminations per sq mm of the leaf surface. It is the ultimate free termination of a veinlet or branch of a veinlet. It is counted in the same preparation as for vein-islet number. The total number of vein-islets and veinlet terminations in four adjoining squares are divided by four in order to get the value in 1 sq mm. The range and average is determined in 10 sets of 2 mm×2 mm area. Observe under 10X (eye piece) and 4X (objective).

Determination of leaf Constants Refer Appendix IV

d) Quantitative microanalysis of processed herbs

Foreign Organic Matter is determined by Lycopodium spore method

Lycopodium spore method: Lycopodium spores are from club moss, *Lycopodium clavatum* Linn. (Lycopdodiaceae). The spores are yellow in colour, spheroidal tetrahedral in shape with reticulate surface. They have uniform average diameter of 25 microns.1 milligram contains average of 94000 spores. They have uniform moisture content hence the weight remains the same and are resistant to pressure. That is why these spores are used to evaluate powdered drug by comparison.

Limitations

i. First the presence of foreign matter has to be established.

ii. Quantitative values for the pure sample need to be determined.

iii. The drug should possess characteristic identifiable particle and distinct from others and resistant to chemical treatment as below:

a) *Discrete particle uniform in size and shape* like starch granules, crystals, etc. In this the weight of particle is proportional to the number.

b) *Particles composed of a layer of cells, one cell thick,* same type of cells, like epidermis, layer of sclerenchymatous cells etc. In this the weight of particle is proportional to the sum of their areas.

c) *Particles having rod-like shape, the length fairly uniform thickness* like ribs of trichomes (Nux vomica). In this the weight of particle is proportional to the sum of their lengths.

This method was introduced by Wallis and accepted as official method Indian Pharmacopoeia (IP, 1966) to determine percentage adulteration in powder drugs. If the results are to be used as standard correction for moisture should be applied.

Determination of foreign organic matter by Lycopodium spore method Refer Appendix V

e) Microchemistry of herbs and processed herbs

Microchemistry is the specific tests carried out on sections of the herbs/ micro-quantities of powder/drops of extracts with required reagents on slides. Colour change or precipitate formation or crystal formation is observed with naked eye or with the help of microscope.

*Micro-sublimation***:** Some powders give sublimates on heating like anthraquinones. Take 50 mg of powder in a watch glass. Place this on a warm hot plate and immediately place a micro-slide on top of the watch glass. A sublimate is formed on the lower surface of the slide.

Micro- extraction and micro- filtration.Take a small quantity of the powder herb on a micro-slide. Mix it with a drop of solvent and place a cover-glass. Leave it aside for two minutes. Take a filter paper strip cut a triangle with the base equal to the width of the cover-glass (18mm) and the two other sides drawn into tapering cone 4 cm long. Soak this in solvent and drain excess. Place the filter paper touching the right hand side edge cover glass and hold the micro-slide in a slant position above another micro-slide. Add solvent drop by drop to the drug under cover-glass from the left side. The solvent passes through the drug and extracts the constituents and passes through the filter paper by capillary attraction. It filters through and collects as drops on the slide kept below. Discard the first colourless drops and collect the drops of extract on different slides (3-4) for testing or a small test tube.

Clarification of microscopic particles

The presence of certain cell contents, such as starch grains, aleurone grains, plastids, fats and oils, may render sections non-translucent and obscure certain characteristics. Reagents that dissolve some of these contents can be used in order to make the remaining parts stand out clearly or produce a penetrating effect. This renders the section more transparent and reveals details of the structures.

If the refractive index of the clarifying agent is close to that of the cell structure, the material being tested becomes almost invisible; if it differs appreciably, the material becomes markedly evident.

The most frequently used clarifying agents are described below:

Chloral hydrate: On gentle heating chloral hydrate dissolves starch grains, aleurone grains, plastids, volatile oils, expand collapsed and delicate tissue without causing any undue swelling of cell walls or distortion of the tissues. It has a refractive index of 1.44-1.48. It is the best reagent for rendering calcium oxalate clearly evident and is particularly useful for small crystals. However, when allowed to stand, it slowly dissolves calcium oxalate, owing to an increase in acidity.

Lactochloral: This has a similar use to chloral hydrate, is usually applied to sections that are difficult to clarify. It may be used cold. Before use, any air present in the specimen should be removed by placing in a dessicator and applying a vacuum.

Lactophenol: It may be used cold or with heating. It has a refractive index of 1.44 and is useful for the preparation of fungi, pollen grains, most non-oily powders, and parasites such as mites and nematode worms. Sizes of starch grains can be measured accurately, but the concentric rings are usually invisible when prepared in this reagent. Crystals of calcium oxalate are clearly visible in lactophenol and shine brightly when illuminated with polarized light. This reagent dissolves calcium carbonate deposits with a slow effervescence, owing to the presence of lactic acid.

Sodium hypochlorite: This is used for bleaching deeply coloured sections. Immerse the sections in the solution for a few minutes until sufficiently bleached, wash with water and prepare the mount with glycerol/ ethanol. The bleached sections give a negative reaction to lignin.

Solvents for fats and oils: Xylene R and light petroleum R can be used to remove fats and oils from oily powders or sections. When necessary, immerse the material in the solvent for a short time, decant the liquid and wash the material with fresh solvent.

Histochemical detection of cell walls and contents

Reagents can be applied to a powdered sample or a section on a slide by the following methods:

Add drops of the reagent to the sample and apply a cover-glass, then irrigate using a strip of filter-paper as described below.
Place drops of the reagent on the edge of the cover-glass of a prepared specimen. Place a strip of filter-paper at the opposite edge of the cover-glass to remove the fluid under the cover-glass by suction, causing the reagent to flow over the specimen.

Using the second method, the progress of the reaction may be observed under a microscope. Care should be taken to avoid using reagent or vapours that could attack the lenses or stages of the microscope.

Cellulose cell walls: Add 1-2 drops of iodinated zinc chloride and allow to stand for few minutes; alternatively, add 1 drop of iodine (0.1 mol/l) VS, allow to stand for 1 minute, remove excess reagent with a strip of filter-paper and add 1 drop of sulfuric acid (~1160 g/l) ; cellulose cell walls are stained blue to blue-violet. On the addition of 1-2 drops of cuoxam , the cellulose cell walls will swell and gradually dissolve.

Lignified cell walls: Moisten the powder or section on a slide with a small volume of phloroglucinol and allow to stand for about 2 minutes or until almost dry. Add 1 drop of hydrochloric acid (~420 g/ l) and apply a cover-glass; lignified cell walls are stained pink to cherry red.

Suberized or cuticular cell walls: Add 1-2 drops of sudan red and allow to stand for a few minutes or warm gently; suberized or cuticular cell walls are stained orange-red or red.

Aleurone grains: Add a few drops of iodine/ethanol; the aleurone grains will turn yellowish brown to brown. Then add a few drops of ethanolic trinitrophenol; the grains will turn yellow. Add about 1 ml of mercuric nitrate and allow dissolving; the colour of the solution

turns brick red. If the specimen is oily, render it fat-free by immersing and washing it in an appropriate solvent before carrying out the test.

Calcium carbonate: Crystals or deposits of calcium carbonate dissolve slowly with effervescence when acetic acid (~60 g/l) or hydrochloric acid (~70 g/l) is added.

Calcium oxalate: Crystals of calcium oxalate are insoluble in acetic acid (~60 g/l) but dissolve in hydrochloric acid (~70 g/l) without effervescence (if applied by irrigation the acid should be more concentrated); they also dissolve in sulfuric acid (~350 g/l), but needle-shaped crystals of calcium sulfate separate on standing after about 10 minutes. In polarized light, calcium oxalate crystals are birefringent. Calcium oxalate is best viewed after the sample has been clarified (e.g. with chloral hydrate).

Fats, fatty oils, volatile oils and resins: Add 1-2 drops of sudan red and allow to stand for a few minutes or heat gently, if necessary. The fatty substances are stained orange-red to red. Irrigate the preparation with ethanol (~750 g/l) and heat gently; volatile oils and resins dissolve in the solvent, while fats and fatty oils (except castor oil and croton oil) remain intact.

Hydroxyanthraquinones: Add 1 drop of potassium hydroxide (~55 g/l); cells containg1, 8-dihydroxyanthraquinones are attained red.

Inulin: Add 1 drop each of 1-naphthol and sulfuric acid (~1760 g/l) ; spherical aggregations of crystals of inulin turn brownish red and dissolve.

Mucilage: Add 1 drop of Chinese ink to the dry sample; the mucilage shows up as transparent, spherically dilated fragments on a black background. Alternatively, add 1 drop of thionine to the dry sample, allow to stand for about 15 minutes, then wash with ethanol (~188 g/l); the mucilage turns violet-red (cellulose and lignified cell walls are stained blue and bluish violet respectively)

Starch: Add a small volume of iodine (0.02 mol/l) ; a blue or reddish blue colour is produced. Alternatively, add a small volume of glycerol/ ethanol and examine under a microscope with polarized light; birefringence is observed giving a Maltese cross effect with the arms of the cross intersecting at the hilum.

Tannin: Add 1 drop of ferric chloride (50 g/l); it turns bluish black or greenish black.