Appendix I

Microscopic Sections of the herb

Preliminary treatment

Redydrate or boil the representative sample of soft tissues like leaves and flowers for 5 minutes.Soakhard and thick herbs like barks, woods, roots, rhizomeandseeds usually soaked in water for 20-30 minutes or more till the tissue become soft.

Sections cutting

Cut representative pieces of the herb from the end which is softened and smoothedinto suitable lengthsand prepare cross or transverse sections by cutting with razor blade or microtome at a right angle to the longitudinal axis of the material. Prepare longitudinal sections by cutting in parallel with the longitudinal axis, either in a radial direction (radial section) or in a tangential direction (tangential section).

Cut the thickandsoftenedtissue by holding the herb in the left hand vertically between the thumb and index finger, keeping the index finger higher than the thumb to give support to the razor blade while cutting. Hold the razor blade horizontally with few a drops of water over the right hand index finger folded in and press it down with the thumb. Microtome can be used. Very small and soft tissues need support and require pith or potato cubes for cutting sections. Cut thin and even sections as possible. Place the sections immediately with a brush moistened with water to a dish containing water.

Flat, small and spherical seeds and fruits may be inserted into a notch cut into a small rubber stopper or embedded in hard paraffin (paraffin wax) as follows. Prepare a hard paraffin block, rectangular in shape, measuring about 7×7×15 mm, and melt a small hole in the centre of one end using a heated needle or thin glass rod. Press the material, which should be dry or softened by exposure to moisture, into this hole. Then prepare sections with a microtome. For the examination of mucilage, aleurone grains or spherical aggregations of inulin, cut the material without using water.

Observe one of the sections under microscope. If the vessels and fibres show a round outline the section is a correct T.S. If the vessels show spiral or reticulate thickenings and fibres as long rod like structures, the section is oblique. In case ofoblique sections, place the material on a micro slide and give a cut at right angles to the axis of growth and then continue to take sections. Pick up thin transparent sections.

Preparation of specimens

Select 1-2 thin sections and transfer them to a slide with the help of a brush.Add 2 drops of chloral hydrate and warm for a fraction of a minute by passing the slide over 3-4 cm above the

spirit lamp flame. If bunsen flame,take care not to boil. Repeat the above step till the section is clear and transparent. Cool the sideandadd a drop of phloroglucinol and allow it to evaporate, followed by aadrop of concentrated hydrochloric acid.Add two drops of glycerine solution to the section. Hold the slide with the left hand slightly slanting towards left. Hold the cover glass in the right hand horizontally, with the thumb and index finger.Place one edge of the cover glass touching the liquid on the slide forming a line along the edge. Then slowly drop it on the section to avoid any air bubbles.Use blotting paper to clean the excess liquid. Do not press the cover glass.All lignified tissues take pink stain. The lignified tissues may be disintegrated as follows:

Cut the material into small pieces about 2 mm thick and 5 mm long, or into slices about 1 mm thick (tangential longitudinal sections are preferred for woods or xylem from stems).

Use one of the following methods depending on the nature of the cell walls. For tissues with lignified cell walls use either method 1 or method 2. For tissues where lignified cells are few or occur in small groups, use method 3.

Method 1.*Nitric acid and potassium chlorate*

Place the material in a test-tube containing about 5 ml of nitric acid (~500 g/l) TS and heat to boiling. Add a small quantity of powdered potassium chlorate R and allow toreact, warming gently if necessary to maintain a slight effervescence; add fresh quantities of powdered potassium chlorate Ras needed. When the tissue appears to be almost completely bleached and shows a tendency to disintegrate, apply pressure with glass rod to the material. If the material breaks, interrupt the reaction by pouring the contents of the test-tube into water. Allow the material to settle, decant it and wash it with fresh water until the acidity is removed. Transfer the material onto a slide and tease it out with a needle. Add 1 drop of glycerol/ ethanol TS and apply a cover-glass. The disintegrated material gives a negative reaction for lignin.

Method 2. Nitric acid and chromic acid:Place the material in a small dish and heat with nitro-chromic acid TS until the material breaks easily when pressure is applied with a glass rod. Wash the material repeatedly with water and transfer onto a slide. Tease out the material, add 1 drop of glycerol/ ethanol TS and apply a cover-glass. The disintegrated material gives a negative reaction for lignin.

This treatment can also be carried out on a slide. Place a moderately thick section of the material on a slide, add the reagent and allow it to stand for about 20 minutes. Separate the cells by applying gentle pressure, or with a sliding movement of the cover-glass. This process is especially useful when the disintegration of the tissues of a section under the microscope needs to be observed to ascertain where isolated cells come from.

Method 3. *Caustic alkali method***:** Place the material in a test-tube containing about 5 ml of potassium hydroxide (~110 g/l) TS or sodium hydroxide (~80 g/l) TS, and heat on a water- bath for 15-30 minutes until a portion breaks easily when pressure is applied with a glass rod. Decant

the liquid and wash the softened material several times with fresh quantities of water. This method is particularly useful for the disintegration of bark, seeds, leaves and flowers, faciliting the elimination of fibres, sclereids, lactiferous tissues and epidermis. The disintegrated material gives a negative reaction for lignin.

Study of sections

As per the morphological group of the herbal material, Transverse (T.S.), Radial Longitudal (R.L.S.) and Tangential longitudinal sections (T.L.S.) are to be studied.Use 100 magnification for sections and 400 magnification for detailed study of cells in different regions, starch and pollen grains, trichome walls and pits on fibres.

Appendix II

Preparation of Powders

Clean the drug to be powdered. Remove all the different parts associated with the herb, even if they are officially permitted like stalks in Senna and Cloves, calyx in Capsicum ., to get the correct identification of characters.Cut larger pieces into smaller pieces and grind the material in an iron mortar and pestle/ lab grinder. Pass through a sieve to obtain a uniform moderately fine powder (44/85). The hard drugs like woods and barks require finer powder than soft drugs, like leaves and flowers.Drugs with high oil content may be treated with petroleum ether to remove oil, dried and the powdered.Continue powdering till all the particles pass through the desired sieve including the ‘tailings’. These are usually the hard tissues and discarding these removes some of the very important characters of identification.Label with the common and botanical name of the drug. Store them in air tight bottles and keep them in a dark, cool place to prevent deterioration and colour change.Separate out small quantities for study. Never transfer back the powders once they are taken out of the containers.

Appendix III

Fluorescence characteristics

Take about 0.5geach of plant powder into clean and dried test tubes. To each tube add 5ml of different organic solvents like distilled water, acetone, ethanol, benzene, chloroform, diethyl ether, methanol, glacial acetic acid, sulphuric acid, nitric acid, hydrochloric acid, 5% $FeCl_3$, 5% I_2, picric acid, 1N NaOH and 1N NaOHandmethanol separately. Shake and allow to stand for about 20-25 minutes. Observe the solutionsunder the visible day light UV light 254 nmand 365 nmfor their characteristic color.

Appendix IV

Determination of Leaf Constants

Equipment

Stage micrometer
This is a glass slide containing a calibrated scale engraved on a small square or round glass. In this 1.0 mm is divided into 10 large units of 0.1mm each (longer lines) numbered 1 to10. Each unit shows further division into 10 smaller units of 0.01mm smaller lines, i.e millimicron. This is used to calibrate the eye piece micrometer.

Eyepiece micrometer
This is an arbitrary scale of 100 divisions engraved on a thick, small, glass disc which fits into the eye-piece. The width of each eye-piece micrometer division is calibrated against the stage micrometer, when required, for different magnifications. These are preferably used along with a microscope mounted with a mechanical stage. Two types of apparatus are used in quantitative microscopy to trace magnified image of the object under the microscope on the paper with pencil kept at 45 degree angle on the working bench.

Swift-Ives Camera Lucida
This is a compact apparatus supplied in a box for careful storage. In this, the image is reflected by the prisms encased in the apparatus, making the image formed in the microscope and the image of paper and pencil placed on the right side of the microscope, on which the image is to be drawn seen super imposed on one another. Then the image can easily be traced on the paper. This needs an adjustable drawing board to adjust the angle in order to avoid distortion while drawing and the field of view is limited and needs adjustment.

Abbe's Drawing Apparatus
In this a plane mirror is attached to a side arm which makes 45 degree angle to the bench surface. The light from the drawing paper, pencil and light from the image to be drawn can be seen through the eye-piece simultaneously. This does not require adjustable drawing board, gives a large field of view.

Mechanical stage
A graduated scale in centimeters on the vertical and perpendicular plane directions of the stage of microscope

Counting-field finder
In case, mechanical stage is not available, this can be used in 'Lycopodium spore method' to move the slide to different positions.

Counting Square
A diagram to guide pre-determined positions to take readings for 'Lycopodium spore method' This prevents the repetition of counting the same fields.

Stomatal number and Stomatal index

Stomatal Index

Preparation of lamina

Take a mature leaf. If the leaf is small, the whole leaf may be taken and if the leaf is large cut 5mm square pieces from the middle portion between the lamina and midrib.

Fresh leaf

1. Peel off the epidermis in thick leaves by breaking into pieces by sheering action. Separate epidermis and clear with it with chloral hydrate.
2. Cut number of 5mm pieces from the middle portion between the lamina and midrib. Boil with chloral hydrate in a test tube placed on a water bath to separate the epidermis out. Carefully place it on a slide with the help of camel hair brush along with 1-2 drops of chloral hydrate cool and then place a cover glass.

Preparing an imprint of the epidermis

Take a little piece of gelatin gel (50%) with the help of a needle. Smear it on a hot slide, place a fresh leaf and slightly press the leaf. Invert the slide and cool it under a tap till the gel is solidified. Then the leaf is removed. This leaves an imprint of the stomata epidermal cells.

Dry leaf

1. Heat the leaf with chloral hydrate in a test tube on a water bath for 30 minutes.
2. Cut the leaf into two pieces, observe under the microscope to see whether the stomata are present on both surfaces and the epidermal cells are easy to trace.
3. Place the cleared leaf with the veins facing down. Then the upper epidermis will be visible.
4. Place the other half with veins facing upwards. Then the lower epidermis will be visible
5. Add two drops of glycerin and place a cover glass.
6. Label the slides as 'upper' and 'lower'.
7. Repeat the procedure with the second half, this time placing the veins side down and take out the lower epidermis.

If the leaf is too thick and dark, separate the epidermis for tracing of the cells as follows:

1. Place one half with veins facing upwards after clearing.
2. Carefully scrap off the upper tissues with the edge of a razor blade, without disturbing upper epidermis. Clean it with brush dipped in chloral hydrate solution. The layer of cells remaining on the slide is the upper epidermis. Turn the layer upside to trace the cells.
3. Repeat the procedure with the second half, this time placing the placing the lower surface facing downwards.

Note: Usually herbs and small shrubs have stomata on both surfaces. In tree species stomata are present on the lower surface. More stomata are present on the lower surface in dorsiventral leaf, almost the same number in isobilateral leaf.

Tracing of cells

1. Draw a square of about 8-10 cm square on a drawing sheet or any unit area.
2. Place the prepared slide on the stage of the microscope.
3. Focus epidermal cells and the stomata first with 10 X10, followed by 10x40 or 10x20.
4. Trace the stomata and the epidermal cells in the square with the help of camera lucida and using suitable magnification
5. Trace epidermal cells and the stomata outside the square to completion on two adjacent sides of the square.
6. Number the complete epidermal cells and the stomata within the square.

7. Continue numbering the cells that are more than half on two adjacent sides of the square.
   8.Calculate the Stomatal index (S.I) = number of stomata/number of stomata plus number of epidermal cells x100

About 10 readings or count of 400 cells are taken to determine the average Stomatal Index.

Stomatal Number

Follow the procedure as given in Stomatal index

Tracing of cells

The number has to be determined per square millimeter.

1. Adjust the drawing board, if swift camera-lucida is used. It is not necessary to adjust angle with Abbe's camera lucida.
2. Draw a line of 1 mm using 10 x10 magnification on a drawing sheet and draw a square on that line with the help of stage micrometer.
3. Replace the stage micrometer with a prepared slide of the leaf
4. Mark the number of stomata in the square with the help of Camera lucida
5. Count the stomata. That gives the number of stomata per sq.mm.
6. Take 25 readings and calculate the average.

(Note the side, for which the stomatal number is determined)
Direct counting of stomata can also be done by squared eye piece, if the drawings are not required

Palisade ratio

Preparation of lamina

1. Take a fresh leaf/dried leaf/ powder. Cut about 5mm square from the lamina between the midrib and the margin of the leaf. If leaf is very small take the whole leaf.
2. Clear the leaf or powder with drop-wise addition of chloral hydrate solution. Warm it on a spirit lamp adding the solution from time to time till the lamina/powder is transparent. Do not boil.
3. Always keep the slide moist. Allow the slide to cool.
4. Place a cover glass. Do not place the cover glass when the slide is warm. (This causes haziness due to presence of vapour below the cover glass, making it difficult to trace).

Tracing of cells

1. Place the prepared slide on the stage of the microscope fitted with camera lucida. Adjust the drawing board and secure a drawing paper with drawing pins/cellotape.
2. Focus on the upper most layer position 1 of the leaf with10X10 first and adjust the focus to see stomata and epidermal cells.
3. Adjust the focus with fine-adjustment screw and suitable magnification..
4. Control the light by adjusting the condenser and mirror. In bright light the cell walls are not visible.
5. Select 4 or 5 groups of epidermal cells. *Avoid stomata or epidermal cell with trichome*.

6. Fix the slide firmly. Do not change the position till epidermal cells and the palisade cells below them have been traced.
7. Trace 4 or 5 epidermal cells with pencil on the paper. Darken the outlines of these cells without moving the paper.
8. Using fine adjustment, lower the focus to palisade cells. Less light may be needed at this stage. Initially the cells will be seen as small rounds loosely packed. Lower the focus till the cells look bigger and compactly packed.
9. Trace the palisade cells under the 4 or 5 epidermal cells selected, but complete the palisade cell even if it falls outside the outlined epidermal cells.
10. Take 4 epidermal cells and 25 readings or 5 epidermal cells and 20 readings from different locations to get 100 cell counts for determination.
11. Remove the drawing sheet. Number the palisade cells that completely fall within the outline of the epidermal cells first, continue numbering the palisade cells which are more than half within the outline of the epidermal cells.
12. Calculate and take the minimum, average and maximum values from 25 readings
    Palisade ratio = Number of palisade cells /number of epidermal cells.

    Note: For isobilateral leaf make a note of the upper or lower epidermis drawn

Vein-islet number and Vein termination number

1. Hold the leaf towards a light source and see if the vein islets are complete. If the leaf is small take the whole leaf. If the leaf is big, cut about 4mm squares from the lamina between the midrib and margin.
2. Clear the leaf by boiling with chloral hydrate in a test tube on a water bath till transparent.
3. Stain with phloroglucinol and hydrochloric acid. Veins take a pink stain.
4. Set up the board for drawing and fix the drawing paper with drawing pins. If using Swift camera lucida adjust the angle of the drawing board.
5. Draw 1mm of stage micrometer line on the drawing sheet with 10x10 magnification and extend it to double the length with the help of the stage micrometer and camera lucida.
6. Draw a big square on this line and divide the square into four squares, two above and two below each representing 1mm of the lamina magnified to 100 times(approximately 10cm2).
7. Place the cleared leaf on the microscope and trace the veins and vein endings on the drawing paper.
8. Trace the veins falling beyond the lines of the squares on all sides to complete the islets.
9. Number the complete islets within the squares and then the islets that are half inside the square on two adjacent sides. The number thus obtained divided by four will give the vein islets per one square millimeter.
   Vein-islet number per square mm = Total number of vein-islets in four squares/ 4.

Vein termination number

In continuation to the above experiment, number the vein-endings in the above drawing in each of the vein islets. This gives the number of vein endings per four square millimeters of the lamina.Take the average per one mm.
Vein-islet number per sq.mm= Total number of vein endings divided by 4

Appendix V

Determination of Foreign Organic Matter by Lycopodium spore method

a) Discrete particle uniform in size and shape

Preparation a suspending agent

Fixed oil or suspending agent; glycerin: tragacanth mucilage: water (2:1:2). This keeps the spores and particles in a suspension. Dilution of the suspension should give about10 to 20 spores in a field.

Method

1. Determine the loss on drying for the powder at 105 C.
2. Mix a weighed amount of air-dry powder of the drug and a weighed amount of lycopodium spores in a small watch glass (100mg drug and 50mg lycopodium spores). Mix with a small flexible spatula.
3. Add oil or suspending agent. Mix for 10 min till a smooth paste is obtained.
4. Transfer the suspension to a small glass tube by draining with the help of a glass rod. Add more suspending agent washing down the mixture into the tube. (about 4 ml of the suspending agent is required for 50 mg of lycopodium spores). This should give about 10 to 20 spores when viewed under high power when a drop of the mixture is mounted under a cover glass.
5. Slowly oscillate the glass tube between the two palms without any air bubbles, until the suspension is uniform
6. Take a glass tube with internal diameter of about 2-3 mm and place one drop each on four slides spread the suspension on the slide less than the area of the cover slip. Apply a cover slip and leave the slides on an even surface to settle.
7. Select 25 fields and count the spores and particles in these fields x 4. Take average
8. Make a similar suspension as above and count particles in 25 fields.
9. Require two value to determine adulteration
10. Calculate the percentage of Foreign Organic Matter from the formula given below

Percentage of foreign Organic Matter = 94000 x 100x n x w / s x m x p

m = Weight in mg of the sample, calculated on sample dried at 105.C
w = Weight of in mg of the lycopodium spores
n = Number of particles in 25 fields
s = Number of spores in 25 fields
p= Number of particles in per mg of the pure foreign matter dried at 105.C
94,000 = Number of spores in one mg of lycopodium

B. *Particles composed of a layer of cells one cell thick – same type of cells*

Determination of Epidermal area per gram of powdered senna leaf.

This is carried out with Senna powder as given above. But the powder is cleared with 3 ml of chloral hydrate by heating on a water bath. Drop of suspension should give 10 –20 spores.

1. With the help of counting field count spores present in 25 fields and trace the outlines of a epidermal particles by means of camera lucida using 10 x 40 magnification.
2. Cut out the tracings and weigh.
3. Cut out a 10cm square of the same paper and find the weight,
4. Calculate the area of the tracings from the above and divide the area by 400 square (160,000) to get actual area of the tracings in 25 fields. Calculate the area of the air dried and correct for loss on drying.

C. *Particles having rod-like shape the length fairly uniform thickness - rod like eg: ribs of trichomes (nux vomica)*

In this the weight of particle is proportional to the sum of their lengths. Proceed as in experiment A.

1. Weigh 0.1 g of nuxvomica powder (No 90) and 0.1 g lycopodium spores.
2. Stain: Moisten with 0.5 ml alcohol mix thoroughly incorporate 1 ml of 1.0% safranin in alcohol (50 %) allow to stand for 10 min
3. Add 1ml of HCl to decolourise the non-lignified tissues and addsufficient suspending agent (7volumes of glycrin, 4 volumes of mucilage 5 volume of water).
4. Count no of spores in 24 fields each of two slides and trace the length of ribs in the same field by camera lucida at a definite magnification and measure them on the drawing sheet.
5. Divide the total length in 24 fields in cm by magnification. This gives actual length
6. Find the weight of spores in 24 fields dividing by 94,000 = weight of nuv vomica in 24 fields
7. Calculate the length of ribs per mg of nuxvomuica = actual length / weight of nux vomica in 24 fields.
8. Calculate the percentage of foreign organic matter.