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Three simple methodologies for the microanatomical study of dicot leaf samples

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Abstract
The success of the leaf anatomical research depends on how much the tissue or cell characteristics differentiate under the compound microscope. This demands, careful preparation of specimens including sectioning, staining, mounting, and so forth. This is possible only with a feasible and standardised methodology for specimen preparation. In this paper, we recommend three standard and simple methodologies for the preparation of leaf specimens to explore 90% of the anatomical details of a leaf, under basic lab conditions. Leaf anatomical study include-leaf surface, leaf skeleton, transverse section of leaf midrib, lamina, and petiole. All these procedures were standardized for fresh leaf materials from selected dicot taxa.

Keywords: compound microscope, leaf anatomy, leaf lamina, leaf midrib, leaf skeleton

Introduction
Leaf anatomical studies had wider applications in the field of comparative anatomy, systematics, phylogeny, paleobotany pharmacognosy, phytopathology and economic botany [1, 3]. Therefore leaf anatomical study had relevance in our present-day academic and research field. Like the other plant anatomy branch, the success of leaf anatomical research depends on how much the tissue or cell characteristics differentiate [2, 9] under the compound microscope and the maximum number of measurements and observations from repeated samples [2]. This is possible only with a feasible and standardized methodology for specimen preparation. Based on our study, here we forwarded, three standard and simple methodologies for the preparation of leaf specimens to explore 90% of the anatomical details of a leaf, under basic lab conditions.

Materials and Methods
Leaf anatomical study include-leaf surface, leaf skeleton, transverse section of leaf midrib, lamina, and petiole. The leaf samples were collected from a tropical tree species, *Sapindus trifoliatus* L. The voucher specimens are deposited in the Herbarium of Department of Botany, C M S College, Kottayam with plant accession No: CMS 3116. All the samples were analyzed using a Magnus MLXi Plus microscope equipped with a Magnus camera adapter.

Leaf epidermal surface.
Leaf epidermal study used in plant anatomy to unravel the leaf surface characteristics like nature and distribution of trichomes, nature and distribution pattern of stomata, cuticular patterns, non-living depositions in the epidermis and the epidermal cell shape. Studies based on the Compound microscope (10x, 40x and 100x) reveals all the above-mentioned fundamental details of the leaf surface. The procedure we selected is a modification of Dilcher [5]. For the details of the methodology see Table 1.

Transverse sections of leaf midrib, lamina and petiole.
Microscopic observation of transverse section of leaf lamina, midrib and petiole, are used to unravel both qualitative and quantitative details, including- midrib Vasculature, size and shape of epidermal cells, palisade and spongy cells, cuticle thickness and non-living depositions. For the anatomical study of leaf lamina and midrib, sections from the middle part of the lamina are preferred. For petiole, sections from the distal, middle and proximal end should be taken, because the vascularization of the petiole changes, when it passes through a petiole [10]. Leaf sections for the microscopical analysis were prepared following the methodology mentioned in Table 1.

Preparation of leaf veins
Leaf skeletons are prepared to express the leaf architecture, for the identification of vein orders, size, type of vein ends and areoles [5, 7]. Leaf skeleton preparation and analysis are an essential part of modern and fossil taxonomic studies of flowering plants [7]. For the preparation of a leaf skeleton, the procedure (Table 1) we selected was with a modified procedure of Hickey [7].

Table 1: Methodology for the preparation of leaf samples for anatomical study

Preparation of Leaf Epidermal surface
1. Cut 3cm^2 sized lamina from the middle part of a leaf, including the leaf margin
2. Soak the leaf portions in a concentrated nitric acid for one or two days.
3. The appearance of air bubbles from the leaf surface indicates the readiness of the epidermis to be separated.
4. Transfer the samples to distilled water.
5. With the help of fine forceps and dissection needles, peel off the epidermal layer from the leaf surface.
6. Wash thoroughly and stain with safranin. Mount the section in 50% glycerine.
7. Seal the coverslip with transparent nail polish and store flat.

Preparation of Transverse sections of leaf midrib, lamina and petiole
1. For the anatomical study of leaf lamina and midrib, sections from the middle part of the lamina are preferred. 2. For petiole, sections from the distal, middle and proximal end should be taken. 3. Treat the section in 30% sodium hypochlorite solution (NaOCl) for 3-5 minutes. 4. Wash 3 times –first two wash in distilled water and third wash in 50% ethyl alcohol. 5. Satin the section either in 1% TBO or 1% safranin (prepared in 50% alcohol). The stain was selected depending on the nature of the material. 6. Wash the excess stain in 90% alcohol. 7. Mount the section in glycerine. Seal the coverslip with transparent nail polish and store flat.
Preparation of Leaf veins
1. Immerse the leaf in 5% sodium hydroxide solution (5%NaOH recommended for normal dicot leaf, it varies with the softness of leaf tissue). 2. Keep the leaf at 40-50^0 C in an oven for a period of one to three days (duration depends on the thickness of the leaf. For soft and delicate leaves, 5% sodium hypochlorite solution is preferred instead of 5% NaOH solution. 3. If the color of the solution becomes dark, then it has to be replaced with new. 4. Once the leaf becomes transparent, wash three times with distilled water and brush to remove the adhering tissues. 5. Bleach the leaf skeleton with 5% sodium hypochlorite solution for 5-10 minutes. 6. Wash and remove the bleaching solution. 7. Stain with 1% safranin (prepared in 50% alcohol). Wash in 90% alcohol to remove the excess stain.

Results and Discussion

The leaf epidermal surface prepared here could be used to unravel the leaf surface characteristics like nature and distribution of trichomes, nature and distribution pattern of stomata, cuticular patterns, non-living depositions in the epidermis, and the epidermal cell shape (Fig. 1a). Microscopic observation of transverse section of leaf lamina, midrib, and petiole, were used for preparing both qualitative and quantitative anatomical data (Table 2), including- midrib vasculature, size and shape of epidermal cells, palisade, and spongy cells, cuticle thickness, and non-living depositions (Fig. 1b, 1c). The Leaf skeletons prepared were enough to express the leaf architecture (Fig. 1d), for the identification of vein orders, size, type of vein ends and, areoles [5, 7, 8].

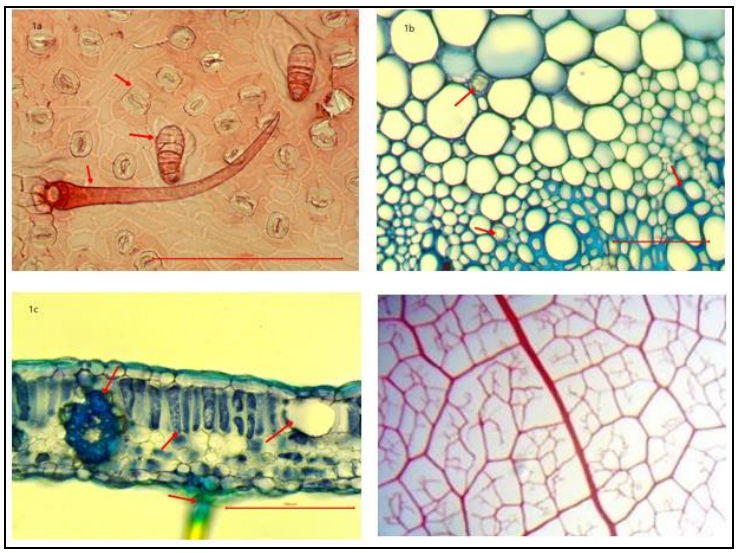

Fig 1: Microscopical view of leaf preparations of *Sapindus trifoliatus*. 1a: leaf surface showing anomocytic stomata, unicellular and multicellular trichomes; 1b: TS of midrib showing xylem vessels, druse crystals and prismatic crystals;1c: TS of lamina showing vein bundle, mucilage cavities and palisade tissue; 1d: leaf architecture showing primary and lateral veins and closed areoles with triveilets.

Table 2: Anatomical Characterization of the leaf components in *Sapindus trifoliatus*

Anatomy characteristics	Leaf lamina	Leaf midrib	Leaf surface	Petiole
Anticlinal wall shape of epidermis	straight	straight	straight	straight
Area of lower epidermal cells (µm)	34.5			
Area of upper epidermal cells (µm)	49.58			
Bifacial mesophyll	+			
Cuticular striations	-	-	-	-
Druse crystals	-	+	+	+
Epidermal striations			-	
Glandular trichome	+	-	+	-
Lamina average thicknes (µm)	60			
Lower epidermis thickness (µm)	4.32			
Mesophyll thickness (µm)	37.612			

Midrib shape	U shaped			
Midrib thickness (µm)	624.71			
Midrib vascular bundle	-	closed, U shaped		
Mucilage cells	+	+	+	+
Multicellular trichome	+	+	+	
Palaside length (µm)	18.462			
Petiole vasculature				crescent, closed
Petiole thickness (µm)	520.44			
Prismatic crystals	+	+	-	+
Secretory idioblasts	-	+	+	+
Size of stomata – length of guard cell (µm)	10.89			
Starch granules	-	+	-	+
Stomata frequancy in lower epidermis	68/mm^2			
Stomatal presence	abaxial			
Stomatal type	anomocytic			
Trichome presence	+	+	+	+
Unicelllular trichome	+	-	+	+
Upper epidermis thickness (µm)	6.58			
Vertically transcurrent vascular bundle	-	-	-	-

- The measured data presented are the mean value of twenty five measurements

Legends - += present, - = absent.

Conclusion

Hope that the leaf anatomical specimen preparation methods, we suggested in this paper could be useful for botany students and scholars to carry out their anatomical study, in a simple but comprehensive way. So that no plant could be kept away from exploring the leaf anatomical diversity.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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