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Pharmacognostical studies in the leaves of *Ceiba pentandra* (L.) Gaertn.

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Abstract
C. pentandra (L.) Gaertn, popularly known as 'kapok', is a medicinal plant with ethnobotanical importance. Morphological characterization of the plant will be useful in its identification. The study of microscopic foliar features revealed anisocytic stomata, glandular hairs and characteristics of veins such as presence of calcium oxalate crystals along the sides of veins, formation of loop by joining of free vein endings and veins covered with parenchymatous bundle sheath. Anatomy of leaf revealed the presence of mucilage cavities, calcium oxalate crystals and starch grains. Histochemical localization of starch, protein, alkaloids, flavonoids, lignin and tannin were performed. Powder microscopy and physicochemical analysis were also done. Phytochemical screening exposed the presence of alkaloids, flavonoids, tannins, steroids, terpenoids, saponins, phenol and resin. The pharmacognostic profile thus developed can serve as a standard for the quality control of *Ceiba* based herbal drugs.

Keywords: *Ceiba pentandra*, pharmacognosy, histochemical localization, phytochemical screening

Introduction
Ceiba pentandra (L.) Gaertn (Family- Bombacaceae) is an emergent deciduous tree of about 50m height. This fast growing tree species is popularly known as 'kapok'. It is usually planted as a wayside or shade tree and is found in the tropical, subtropical and inter tropical regions of the world [1-2]. In traditional medicine different parts of the plant has been in use as diuretic, emetic and antispasmodic [3].The plant is also utilized in the treatment of skin diseases, diabetes, dysentery, eye diseases, insect bite, arthritis, chronic fever, diarrhoea and bronchitis [4]. Pharmacological studies prove that different parts of the plant show anti-inflammatory [5], anti-ulcerogenic [6], hypoglycemic [7], hypolipidemic [8] and hepatoprotective activities [9]. Ethnobotanical evidences claim the use of pounded leaves of *C. pentandra* as a dressing on tumours [10]. The vitamins C and E present in the leaf and bark extract can help to repair the free radical damage to the cells and can be therefore used as a vitamin supplement [11]. The leaves, seeds, bark and resin are utilized in the treatment of asthma, kidney disorder, dysentery and fever [12].The mucilage obtained by boiling the mature leaves is used to remove foreign matter from the eye in Ivory Coast and as an emollient and sedative in Garbon [13].
Pharmacognostic and phytochemical studies are inevitable to avoid chances of adulteration to ensure identity of the plant. Such standardization procedures are relevant to the pharmaceutical industries for quality control and assurance of safety and efficacy of herbal products. The present study is focused on the pharmacognostic characterization and phytochemical evaluation of *C. pentandra* leaves.

Materials and Methods
Plant Material
Ceiba pentandra leaves were collected from Aluva, Ernakulam district of Kerala and was identified at the Silviculture Department, Kerala Forest Research Institute (KFRI), Peechi. The voucher specimen was deposited in the National Herbarium Collection at KFRI with accession No. 13055. Fresh leaves were washed well in running water and were used for macroscopic and microscopic studies. Macroscopic Evaluation Different macroscopic features such as colour, size and shape of leaves, stem, flowers, fruits and seeds were recorded.

Microscopic Evaluation
Leaf
Stomatal type, stomatal index and palisade ratio were determined based on the standard methods of Metcalfe and Chalk (1979) [14], Wallis and Dewar (1933) [15] and Salisbury (1927) [16] respectively. The specimen preparation for scanning electron microscopy (SEM) was based

on modified methodology of Talbot and White (2013) [17]. Leaf clearing, vein islet number and vein let termination number were performed by the methods of Gardner (1975) [18], Levin (1929) [19] and Hall and Melville (1951) [20] respectively. Photomicrographs were taken using LEICA DM 1000 LED microscope and for scanning electron microscopy TESCAN VEGA 3 SBH was used.

Anatomical and Histochemical Evaluation

For qualitative microscopic evaluation, free hand sections of fresh leaves were used. Single staining with safranin and double staining with safranin and fast green were done. Histochemical analysis was carried out using different reagents viz. Lugol's iodine solution for starch (Jensen, 1962), Aqueous NaOH for flavonoids (Johansen, 1940), Biuret method for total proteins (Gahan, 1984), Wagner's reagent for alkaloids (Furr and Mahlberg, 1981), Schiff's reagent for lignin (Mc Lean and Cook, 1941), and hydrochloric - vanillin for tannins (Valette *et al*, 1998) [21-26].

Powder microscopy

The presence of various types of tissues or structures in the crude powder, microscopic observations were performed (Khandelwal, 2008) [27].

Physicochemical Analysis

Moisture content, total ash, acid insoluble ash, water soluble ash and extractive values were calculated as per the methods in Ayurvedic Pharmacopoeia of India (1989) [28].

Preliminary Phytochemical Screening

Dried leaf powder was extracted with various solvents like methanol, acetone, chloroform, petroleum ether and ethyl acetate by cold maceration process. The extracts were concentrated and phytochemical screening was done using standard procedures (Harborne, 1984) [29].

Results

Macroscopic Evaluation

Ceiba pentandra L. Gaertn is a large tree with straight and cylindrical trunk. It bears horizontal branches that were arranged in whorls (Fig. 1A). Outer surface of the bark was grey and spineless. Old trees developed plank like buttresses at the base (Fig. 1B). The palmately compound leaves were alternately arranged and gathered towards the apex of branchlets and bear 5 to 9 leaflets (Fig. 1C). The long glabrous petiole was 5 – 25 cm long, reddish in colour towards the base and pulvinated on both ends. The leaflets were 5-15 cm long with a short petiolule, glabrous, elliptical to oblanceolate with entire margin and acuminate apices. Flowering occured massively during the dry period of February to March, when the trees were devoid of leaves. The inflorescence were fascicles, borne mainly at the ends of branches or in axillary position (Fig. 1D). Flowers were formed in clusters of 25-30 and were usually inclined or pendant in nature. Pedicels were glabrous and 2-4 cm long. The calyx was green, persistant, 4-5 lobed and campanulate. The 5 petals were creamy white and tomentose on the outer side. There were five staminal filaments ending in twisted anthers. Ovary was pyriform and stigma was exerted above the stamens. Fruits were ellipsoid to fusiform capsules and tapered towards both ends (Fig. 1 E). They were 10-25 cm long and 5-8 cm in diameter, green when young and become brown on maturity. When mature, they split into 5 valves and released the characteristic "silk cotton'' (Fig. 1F). Numerous black seeds with copious white silky fibres were freely dispersed by wind.

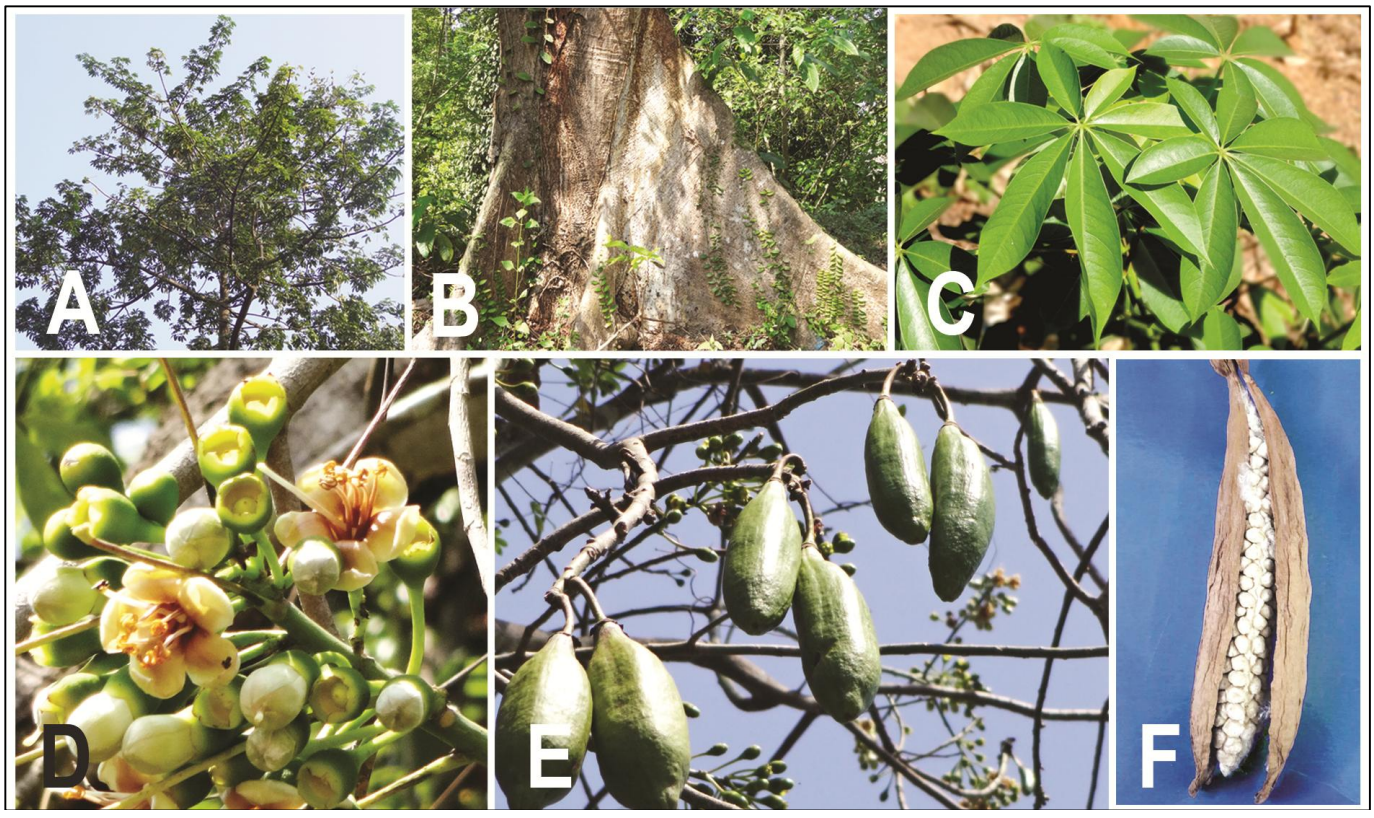

Fig 1: Morphological features: (A) Plant; (B) Buttress; (C) Leaf; (D) Inflorescence; (E) Young fruit; (F) Mature fruit split open with white fibres.

Microscopic Evaluation

Foliar Features

Stomata were absent in the upper epidermis, whereas lower epidermis contained numerous anisocytic stomata along with few glandular hairs. The hairs possessed uniseriate stalk and multicellular head and were placed in a small depression on the lower epidermis. The photomicrographs of stomata and hairs are given in Fig.2A-D. The stomatal index and palisade ratio were 16% and 5.5 respectively. The leaf was pinnately veined and vein islets were variable in size and shape. The peculiar features observed in the venation pattern include presence of calcium oxalate crystals along the sides of veins, formation of loop by joining of free vein endings and veins covered with parenchymatous bundle sheath. The vein islet number and vein termination number were found in the range of 8-10 and 4-6 respectively. The photomicrographs of leaf vein is shown in Fig.2E-F.

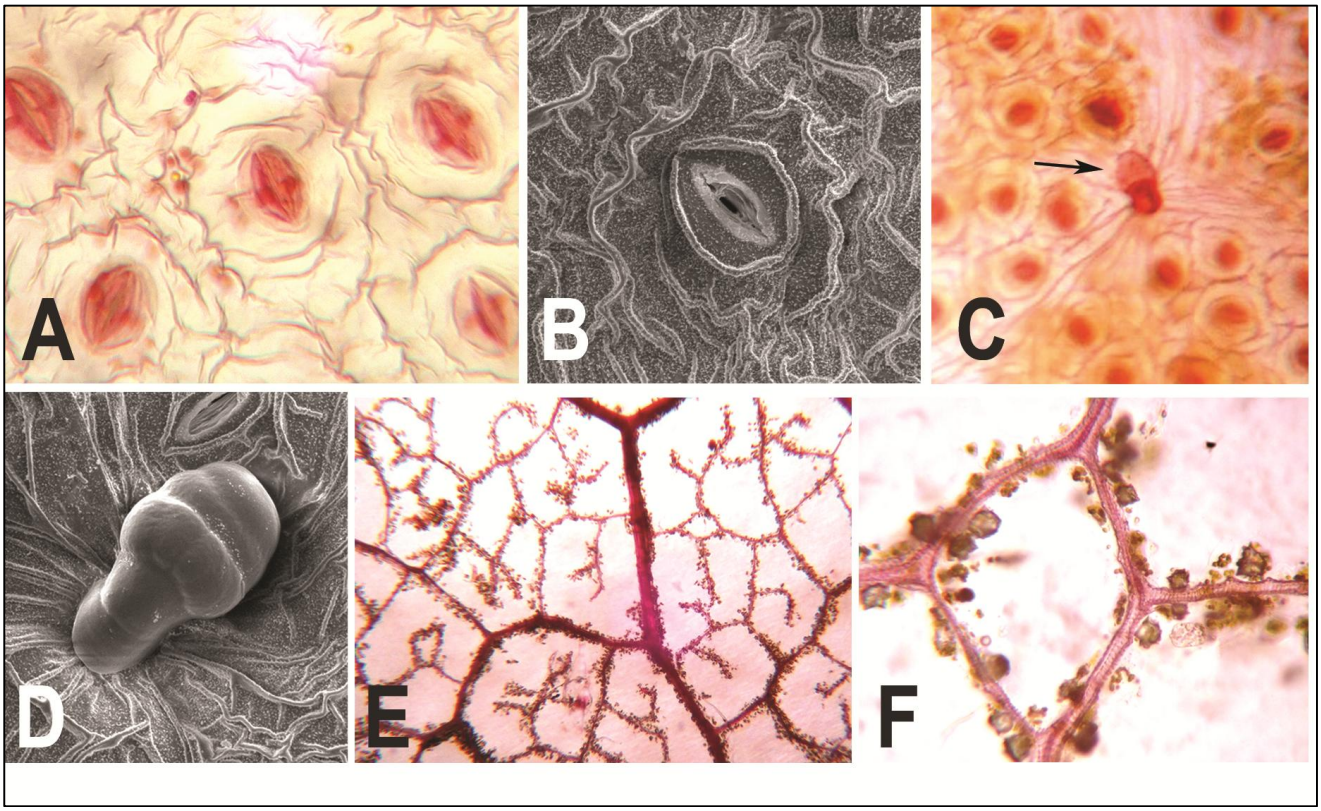

Fig 2: Foliar features: (A) Anisocytic stomata; (B) SEM image of stomata; (C) Glandular trichome; (D) SEM image of glandular trichome; (E) Leaf venation pattern; (F) Loop formed by vein endings

Anatomical and Histochemical Evaluation

Leaf

The transverse section of leaflet was dorsiventral in nature (Fig.3A). The upper epidermis was formed of single row of thin walled polygonal cells with thick striated cuticle (Fig.3B). Hairs and stomata were absent in the upper epidermis. The lower epidermal cells were polygonal and thin walled covered with thick cuticle. It showed anisocytic type of stomata and few glandular hairs. Mesophyll was differentiated into upper palisade and lower spongy tissue (Fig.3D). The palisade was composed of one layer of columnar cells and was interrupted in the midrib region by lignified parenchyma cells. The spongy tissue consisted of thin walled, irregular chlorenchymatous cells with numerous intercellular spaces. Calcium oxalate crystals were present in the spongy tissue. In the upper part of midrib, cortical tissue was formed of 6-8 layers of lignified parenchyma followed by a region with mucilaginous cavities and then by 2-4 layers of lignified cells with few calcium oxalate crystals (Fig.3B). In the lower part of midrib, there were 3-4 rows of collenchyma with mucilaginous cavities in between. Few of those cells also contained calcium oxalate crystals (Fig.3C). It was followed by parenchyma cells which surrounded the vascular system. The parenchyma layer close to the pericycle contained starch grains and calcium oxalate crystals. Pericycle consisted of 4-8 layers of lignified fibres that form an arc above the vascular bundles. Vascular system in the midrib region consisted of crescent shaped, dissected vascular bundle which was accompanied by separate, inverted smaller bundles and each of them was again surrounded by pericycle. Xylem was traversed by 1-4 rows of medullary rays in between. Phloem consisted of thin walled elements. In the lower region, intraxylary phloem was present in between xylem and pericycle which was intersected by lignified cells.

Petiole

The transverse section of petiole was wavy in outline. The outer epidermis consisted of polygonal cells covered by striated cuticle. Inner to the epidermis was 1-2 layered parenchymatous hypodermis with few calcium oxalate crystals. It was followed by cortex which was divided into three regions (Fig.3E). The outer 3-5 layered collenchymatous region was accompanied by mucilaginous cavities followed by 2-4 layers of parenchyma cells containing calcium oxalate crystals (Fig.3F). Parenchyma was followed by lignified pericycle of 4-8 layers. The cells of pericycle were intersected by lignified parenchyma cells. Vascular system consisted of dissected bundles and was surrounded by a continuous ring of pericycle. Radiating medullary rays were seen traversing through the xylem. Between the xylem and pith, intra-xylary phloem was present. Pith consisted of parenchyma cells with large intercellular spaces.

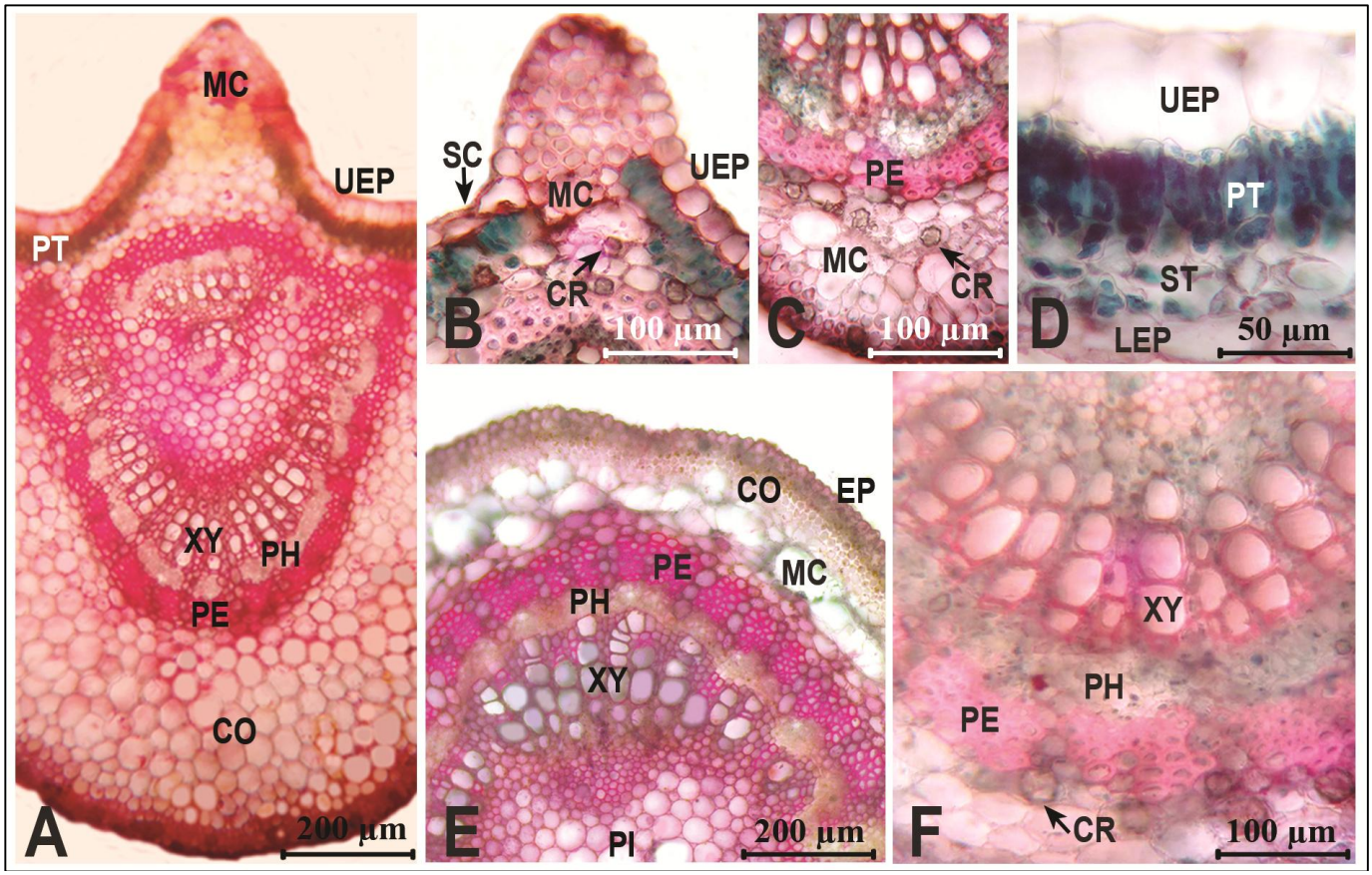

Fig 3: Leaf anatomy: (A) Midrib cross section; (B) Upper part of midrib; (C) Lower part of midrib; (D) Transverse section of lamina; (E) Transverse section of petiole; (F) Portion of petiole magnified. Abbreviations: EP- epidermis, UEP- upper epidermis, LEP- lower epidermis, SC- striated cuticle, MC- mucilage cavity, PT- palisade tissue, ST- spongy tissue, CR- crystals, CO- cortex, PE- pericycle, XY- xylem, PH- phloem, PI- pith

Histo chemistry of Leaf

In histochemical study various phytoconstituents like starch, proteins, alkaloids, flavonoids, tannin and lignin localized in different tissue zones were detected. The results are summarized in Table I and presented in Fig.4 A- L.

Table 1: Histochemical localization

S. No.	Cell contents	Test / Reagents	Nature of changes	Histological location
1	Starch	Lugol's iodine solution	Blue-black coloured globules	Midrib - Parenchyma close to pericycle
				Lamina – Mesophyll tissue
2	Protein	Biuret method	Red colour	Midrib – Few cells of cortical parenchyma
				Lamina –Few cells of palisade tissue and cells of spongy tissue just above lower epidermis
3	Alkaloids	Wagner's reagent	Reddish brown colour	Midrib – Lignified parenchyma cells of cortex and parenchyma cells towards vascular system
				Lamina – Scattered cells of mesophyll tissue
4	Flavonoids	Aqueous NaOH	Wine red colour	Midrib – Lignified parenchyma cells of cortex and parenchyma cells in the centre of vascular system
				Lamina – Palisade tissue and mesophyll cells just above the lower epidermis
5	Tannin	Hydrochloric vanillin	Red colour	Midrib – Few cells of cortical parenchyma and phloem
				Lamina – Few cells of palisade tissue and cells of spongy tissue just above the lower epidermis
6	Lignin	Schiff's reagent	Pink or Majenta colour	Midrib – Pericyclic fibres
				Lamina – Vascular bundle

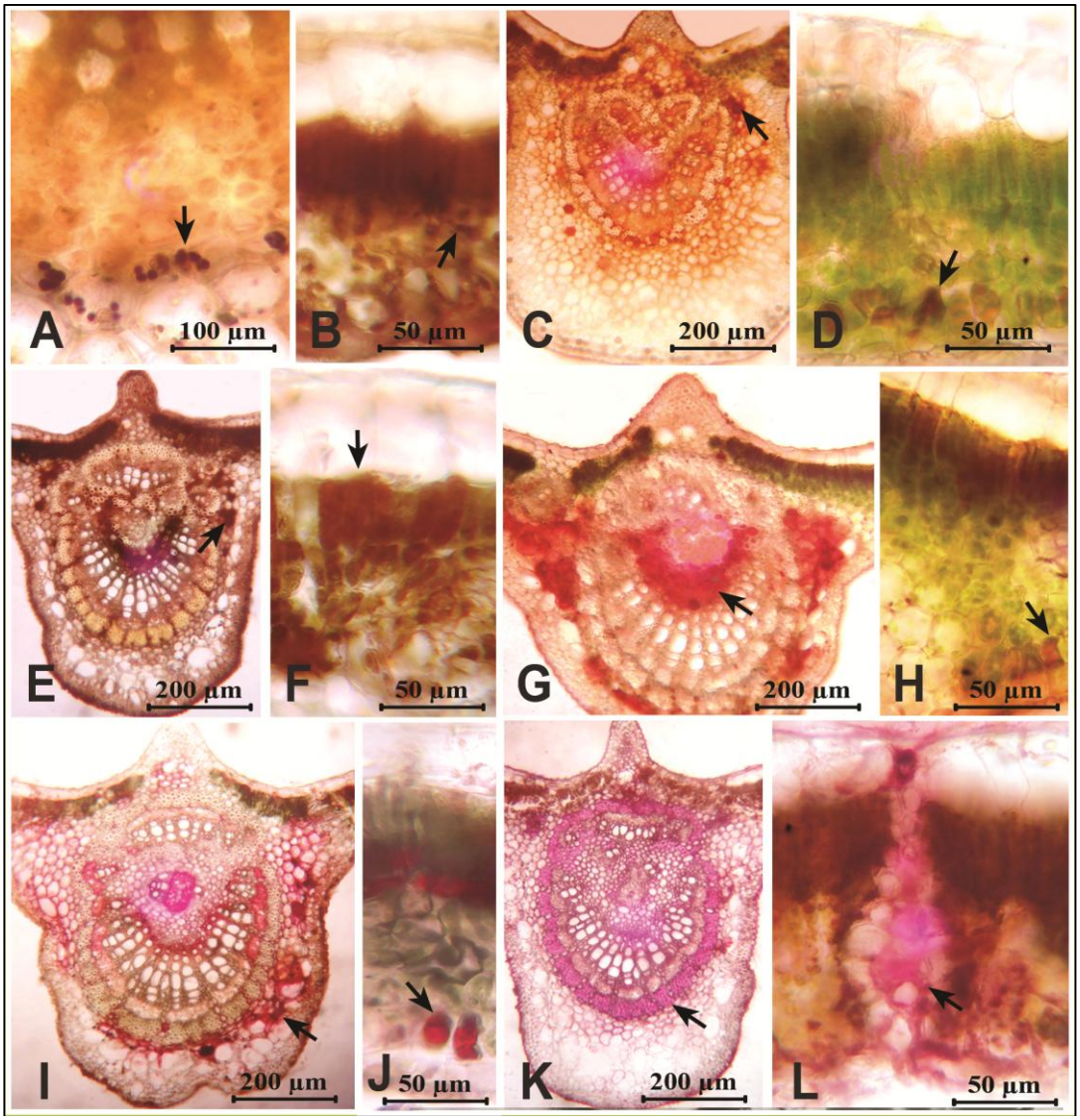

Fig 4: Histochemical localization: (A) Starch grains in midrib; (B) Starch grains in lamina; (C) Proteins in midrib; (D) Proteins in lamina; (E) Alkaloids in midrib; (F) Alkaloids in lamina; (G) Flavonoids in midrib; (H) Flavonoids in lamina; (I) Tannins in midrib; (J) Tannins in lamina; (K) Lignin in midrib; (L) Lignin in lamina.

Powder microscopy

The leaf powder was green in colour with characteristic odour and can be identified by its microscopic features such as fragments of upper epidermis showing polygonal cells with thick striated cuticle, fragments of lower epidermis with anisocytic stomata, calcium oxalate crystals, fragments of pericyclic fibres, fragments of lignified xylem vessels, starch grain, fragments of cortical parenchyma cells with crystals of calcium oxalate and fragments of lignified parenchymatous cells (Fig. 5A-H).

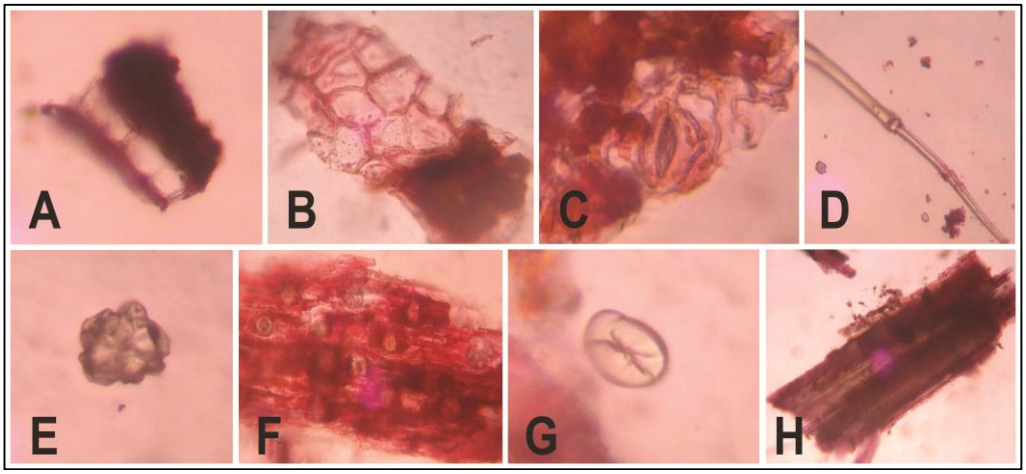

Fig 5: Powder microscopy: (A) fragment of upper epidermis; (B) lignified parenchyma cells; (C) fragment of lower epidermis with stomata; (D) pericyclic fibre; (E) calcium oxalate crystal; (F) cortical parenchyma cells with crystals of calcium oxalate; (G) starch grain; (H) lignified xylem vessel.

Physicochemical Analysis

Physicochemical characterization of leaf powder was done based on the p^H, moisture content, extractive values, total ash, acid insoluble ash and water soluble ash and the results are presented in Table II.

Table 2: Physicochemical parameters

S. No:	Parameters	Result
1	p^H	5.56
2	Moisture content	11.97%
3	Water soluble extractive	13.90%
4	Alcohol soluble extractive	10.87%
5	Acetone soluble extractive	3.83%
6	Ethyl acetate soluble extractive	3.89%
7	Chloroform soluble extractive	5.89%
8	Total ash	8.64%
9	Acid insoluble ash	1.53%
10	Water soluble ash	2.79%

Preliminary Phytochemical Screening

The results of phytohemical screening of various extracts are given in Table III.

Table 3: Preliminary phytochemical screening

S. No.	Phytoconstituent	Petroleum Ether	Ethyl Acetate	Methanol	Acetone	Chloroform
1	Flavonoids	_	+	+	_	_
2	Coumarins	_	_	_	_	_
3	Tannins	_	+	_	_	_
4	Alkaloids(Mayer's)	_	+	_	_	+
5	Alkaloids(Wagner's)	_	+	_	+	_
6	Steroids/Terpenoids	+	_	+	_	_
7	Saponins	_	+	+	_	_
8	Quinines	_	_	_	_	_
9	Anthraquinones	_	_	_	_	_
10	Phenol	_	+	+	_	+
11	Resin	+	_	_	_	_
12	Reducing sugar/Glycoside	_	_	_	_	_
13	Protein	_	+	_	_	_
14	Carbohydrate	+	+	+	+	+

Discussion

In the developed countries the major obstacle for the promotion of alternative medicines is the lack of documentation and absence of strict quality control measures. Hence, there is an urgent need for the documentation and standardization of traditional medicinal plants. The standardization can be achieved through 'pharmacognostic' and phytochemical studies [30]. The foliar epidermal cell characters were utilized in solving many taxonomic problems at different levels of plant taxa [31-34]. Stomatal characters have taxonomic and pharmacognostic value in the identification of plant taxa [35-36]. In *C. pentandra*, the epidermal cells were polygonal and were devoid of hairs and stomata in the upper side. Anisocytic type of stomata was observed in the lower epidermis. The trichome characters were also valuable taxonomic markers which can help in the identification of plant species [37-39]. Few glandular hairs with unicellular stalk and multicellular head were seen on the lower epidermis. In a previous study by Darwish *et al* (2015) in *C. pentandra* var. *pentandra* cultivated in Egypt, similar results were obtained [40]. The formation of loop by joining of free vein endings, veins covered with parenchymatous bundle sheath and presence of calcium oxalate crystals along the sides of veins can be utilized as diagnostic features for the identification of *Ceiba* leaves. Bhat (1995) studied the leaf architecture of 13 species of *Hibiscus* (Malvaceae) and he reported that the primary and secondary veins were covered with parenchymatous bundle sheaths, but loop formation by tracheids were not observed in any of the species [41]. The joining of free vein endings to form loops was reported in the case of *Thalictrum dipterocarpum* [42]. Manokari and Shekhawat (2016) reported the prominent presence of calcium oxalate crystals all along the primary and secondary veins of *Merremia tridendata* [43]. Advances and improvements in the field of microscopy improved the accuracy of botanical identification. In contrast to light microscope, SEM produces a higher resolution and provides three-dimensional images. This can be extensively used for the investigation of surface topology of plant materials namely leaves, pollen grain and seeds [44].In the present study, scanning electron microscope studies conducted in the stomata and trichome has helped in a better understanding of their structure. According to Oliveira *et al* (2012), the vegetative parts of therapeutically important plants must be characterized both anatomically and histochemically [45]. Anatomical studies could reveal the various aspects of secretory structures and secretion of secondary metabolites which will contribute to the correct localisation and extraction of phytochemicals [46]. In the present investigation, the anatomical characterization of different parts of leaves revealed certain diagnostic features like mucilaginous cavities, calcium oxalate crystals, starch grains and dissected vascular bundles surrounded by pericycle. The secretory cells produce mucilage, tannin, essential oils, crystals and resins which may be related with the chemical defence mechanism in plants [47-48]. The characteristics of cell inclusions like aleurone grains, silica and starch granules, cluster crystals and prisms of calcium oxalate are relevant features in the identification of

unorganised crude drugs [49]. Calcium oxalate crystals have an ecological role as they act as static or active defense structures in plants [50]. The synthesis of these crystals were not only influenced by calcium availability but also by herbivory. In the seedling leaves of *Sida rhombi folia* (Malvaceae), the synthesis of crystals is found to increase with herbivory even if there was limited calcium availability [51]. Histochemical localization studies allows a quick and cost effective method for the preliminary evaluation of plant species in the search for new pharmaceuticals [52-55]. The search for novel compounds from plants can be minimised to a great extent by previous histochemical screening thereby reducing the cost for pharmaceutical research [56].From histochemical localization studies it is evident that starch, protein, alkaloids, flavonoids, lignin and tannin are present in detectable amounts in different tissue zones of *C. pentandra* leaves. The cortical parenchyma cells of midrib and mesophyll tissue of lamina were found to be the main sites of synthesis or storage of these phytoconstituents. The classical procedure of powder microscopy is a powerful method for species identification and authentication of the herbal drug [57]. In this study the leaf powder showed the presence of fragments of upper epidermis showing polygonal cells with thick striated cuticles, lower epidermis with anisocytic stomata, calcium oxalate crystals, lignified xylem vessels, starch grain, lignified parenchymatous cells, pericyclic fibres and cortical parenchyma cells with crystals of calcium oxalate. The evaluation of physicochemical parameters is helpful in setting standard for the crude drug as well as in the detection of adulterants [58]. The value of extractable matter varies according to the purity of crude drug and the polarity of solvent used. In the present study, water was found to be the best extractive solvent because it extracted out maximum amount of phytoconstituents from the leaves. The lowest extractive value was shown by chloroform which indicates that comparatively lesser number of phytoconstituents has been leached out from the leaves. The moisture content was related to the stability and quality of crude drugs because if the water content is high the drug will get deteriorated thereby spoiling the biomass and active principles present in it [59].The moisture content was found to be 11.97%.Ash value is an important tool considered as an indicator of the inorganic constituents and other impurities present in the drug. In *C. pentandra* leaves, the values for total ash, acid insoluble ash and water soluble ash were 8.64%, 1.53% and 2.79% respectively. Abou-Elela *et al* (2015) carried out the phytochemical screening of n-hexane and methanol extracts of aerial parts of *C. pentandra* (L.) Gaertn. var *pentandra*. In their investigation steroids, tannins, flavonoids, triterpenes, carbohydrates, saponins, fats, oils and resins were found to be present and alkaloids were absent [60]. But previous studies have mentioned the presence of alkaloids also in *C. pentandra* species [61-64]. The presence of bioactive compounds like flavonoids, tannins, alkaloids, steroids, terpenoids, saponins, phenol, resins, protein and carbohydrates were confirmed in preliminary screening. Similar works were reported in other genera as a tool for the detection of adulteration and authentication of the raw drug [65]. The results obtained in the present study highlight its prospect to be a major candidate for further studies.

Conclusion

Recently there has been an increased interest in the search for medicinally potential natural compounds as they are considered safe with minimal or no side effects which are easily available and affordable. However natural drugs always have chances of adulteration. This commercial practice of substituting and adulterating the genuine herbal drugs is posing great threat to the pharmaceutical industry. In this context, pharmacognostic investigations are relevant in solving problems related to quality, safety and efficacy of herbal products. The pharmacognostical evaluation of *C. pentandra* leaves have given valuable information regarding its morphology, microscopic features, physicochemical characteristics and phytochemical composition. This will be helpful in the identification and assessment of purity of crude drug.

Conflict of Interest

The authors declare no conflict of interest.

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