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ESTABLISHMENT OF PHARMACOGNOSTICAL STANDARDS AND IT'S COMPARATIVE STUDIES ON THE LEAVES OF *Citrus aurantium L.* AND *Citrus sinensis* L. Rutaceae.

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ABSTRACT

Development of pharmacognostical profile of the plant drug would assist in standardization for quality, purity, and sample identification. In that Micromorphological evaluation plays a vital role to distinguish the closely related species. The fresh, powdered, and anatomical sections of the closely related species *Citrus aurantium L.* and *Citrus sinensis* L. belonging to Rutaceae family was carried out to determine the micromorphological, physio-chemical and phytochemical profiles. In the microscopic studies, *Citrus aurantium* leaves showed the presence of several long brachy- sclereids, straight uniseriate xylem

elements and crystals in the form of druses. They are spherical bodies with spiny surface. The druses are solitary in each cell. Whereas *Citrus sinensis* leaves showed the presence of simple brachy sclereids; these sclereids are polyhedral in shape with thick lignified secondary wall and wide canal like simple pits, the vascular strand consists of numerous, long, narrow xylem elements and xylem fibers. The calcium oxalate druses common in the pith cells. Phytochemical evaluation revealed the presence of flavonoids, terpenoids, phenols, glycosides, carbohydrates, steroids, saponins, proteins and amino acids. The results were almost similar in both the drugs. The physio-chemical determination would assist in the estimation of ash content, extractive matters, and moisture content. The results of the study could be useful in setting some diagnostic indices for the identification and preparation of a monograph of the plants chosen.

Keywords: *Citrus aurantium L*. and *Citrus sinensis* L, Rutaceae, Pharmacognostic profile, Physio-chemical evaluation, Phytochemical investigation

INTRODUCTION

Citrus aurantium L. commonly known as bitter orange is widely and easily available plant belonging to the family Rutaceae. It is also known as sour orange, Seville orange, bigarade orange **[1]**. *Citrus aurantium* (Khattak: Hindi, Narangam, Narattai: Tamil) is a tree with greenish white, glabrous shoots which cultivated in India for its fruit and used for various medicinal purposes. Bitter orange is regularly cultivated in Khasi hills and Catcher. It is native to southeastern Asia. It is 3rd most important fruit crop in India. Its ethno-medicinal application has been well known for a long time **[2]**. It is traditionally known to be useful for the treatment of wide panel of diseases like stomachache, vomiting, blood pressure, cough, cold, bronchitis, earache, dysentery, diarrhea, abdominal pain, and fever. Bark used for UTI ailments. Infusion of dried flower is orally used for influenza, insomnia, as a cardiovascular analeptic, anti-spasmodic, for cold, sedative, digestive. Root is used as treat boils and urinary tract infections **[3-5]**.

Citrus sinensis L. commonly known as sweet orange is a small, shallow-rooted evergreen shrub or tree growing about 6 - 13 meters tall with an enclosed conical top and mostly spiny branches **[6]**. A very well-known fruit, widely available in countries around the world. The tree is commonly cultivated for its fruit in warm temperate, subtropical, and tropical zones **[7]**. It prefers a prominent change in the seasons and so is not so suited to the tropics, where it is grown more as a garden tree, but is widely grown commercially in the subtropics **[6]**. Citrus species contain a wide range of active ingredients and

research is still underway in finding uses for them. They are rich in vitamin C, flavonoids, phenolic acids, and volatile oils. They also contain coumarins such as bergapten which sensitizes the skin to sunlight. Bergapten is sometimes added to tanning preparations since it promotes pigmentation in the skin, though it can cause dermatitis or allergic responses in some people **[8]**. The fruit is appetizer and blood purifier. It is used to allay thirst in people with fevers and treats catarrh. The fruit juice is useful in the treatment of bilious affections and bilious diarrhea. The fruit rind is carminative and tonic. The fresh rind is rubbed on the face as a cure for acne. The dried peel is used in the treatment of anorexia, colds, coughs etc., **[9]**. A decoction of the leaves with salt is taken orally for digestive tract ailments, nerve disorders, fever, asthma, blood pressure, general fatigue, and vomiting. The crushed leaves or fruit juice is massaged into the skin to relieve itching. Fruit juice or leaf decoction with sugar is taken orally for cold and loss of appetite, while crushed leaf decoction as a bath relieves headache and rheumatism. Macerated root, leaf or fruit mesoderm is taken orally for urethritis. Macerated fruit mesoderm or bark decoction is taken orally for liver ailments. Broken bones are massaged with roasted fruit. Leaf oil exhibits carminative properties and light antispasmodic and sedative properties **[10-11]**.

MATERIALS AND METHODS

Chemicals

Formalin, acetic acid, ethyl alcohol, chloral hydrate, toluidine blue, phloroglucinol, glycerin, hydrochloric acid and all other chemicals used in this study were of analytical grade and purchased from Asta Tech, U.S.A.

Plant collection and authentication

The leaves of the healthy plants of *Citrus aurantium* L. and *Citrus sinensis*.L. selected for our study were collected from Azhagar hills, Madurai District, Tamil Nadu, India during the month of January 2020 and was authenticated by Dr. P. Jayaraman, Taxonomist, Director of Plant Anatomy Research Institute, Tambaram, Chennai, Tamil Nadu, India.

Macroscopic analysis

Macroscopic analysis of the plants was done by observing its external features. Their shape, size, surface characters, texture, color, odor, taste are identified and compared with reference according to the standard protocol **[12]**.

Microscopic studies

Fixation

Care was undertaken to select healthy plant and normal organs. The required sample of different was cut and removes from the plant and fixed in FAA (Formalin -5ml+ acetic acid 5ml+70%

ethyl alcohol 90ml). After 24 hours of fixing, the specimens were dehydrated with graded series of tertiary butyl alcohol. Infiltration of specimen was carried out by gradual addition of paraffin wax (melting point 58-60ºC) until TBA (Tertiary butyl alcohol) solution attained super saturation. The specimens were cast into paraffin blocks **[13]**.

Sectioning:

The paraffin embedded specimens were sectioned with the hand and dewaxing of section was done by customary procedure. The sections were stained with toluidine blue. Since toluidine blue is polychromatic stain. The staining results were remarkable good and some cytochemical reactions were also obtained. The dye rendered pink color to the cellulose wall, blue to the lignified cells, dark green to suberin, violet to mucilage, blue to protein bodies etc. Wherever necessary sections were also stained with safranin and IKI (Iodine + Potassium iodide) for starch. For studying the stomatal morphology, venation pattern and trichome distribution, Para dermal section (section taken parallel to the surface of leaf) as well as clearing of leaf with the 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffery's maceration fluid were prepared. Glycerin mounted temporary preparation were made for macerated or cleared materials. Powdered materials of different parts were cleared with sodium hydroxide and mounted in glycerin medium after staining. Different cell component was studied and measured **[14-15]**.

Photomicrograph:

Microscopic descriptions of tissues are supplement with micrographs wherever necessary. Photograph of different magnification were taken in Nikon lab photo 2 microscope units. For normal observations bright field was used. For the study of crystal, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under the polarized light they appear bright against dark background. Magnifications of the figure are indicated by the scale bars within the pictures **[15]**.

Analytical Parameters

Ash values, extractive value, loss on drying were used for the study of physical properties.

Ash value

Ash values are helpful in determining the quality and purity of crude drug, especially in the powdered form. The ash of any organic material is composed of their non-volatile inorganic components. Controlled incineration of crude drugs results in an ash residue consisting of an inorganic material (metallic salt and silica). This value varies within wide limits and is therefore, an important parameter for

purpose of evaluation of crude drugs. In certain drugs, the percentage variation of the weight of ash from sample to sample is very small and any marked difference indicates a change in quality. The ash value can be determined three different methods to measure the total ash, the acid insoluble ash and water-soluble ash **[16-18]**.

Determination of total Ash value

Weigh accurately about 5gm of air-dried powdered drug was taken in a tarred silica crucible and incinerated by gradually increasing the temperature to make it dull red until free from carbon cooled weighed then calculated the percentage of total ash with reference to air dried drug.

Determination in of acid insoluble ash value

Boil the total ash with 25ml of 2M HCl for 5 minutes, collect the insoluble matter in a silica crucible or on an ash less filter paper and wash hot water, ignite then cool in a desiccators and weight. Calculate the percentage of acid insoluble ash with reference to the air-dried drug.

Determination of water-soluble ash value

Water soluble ash is that part of the total ash content which is soluble in water. It is good indicator of either previous extraction of the water-soluble salts in the drugs of incorrect preparation. To the crucible containing the total ash, add 25ml of water and boil for 5 minutes. Collect the insoluble matter in a sintered glass crucible or on an ashless filter paper. Wash with hot water and ignite in a crucible for 15 minutes at a temperature not exceeding 450^{o}C. Subtract the weight residue in mg from the weight of total ash. Calculate the content of water-soluble ash in mg/g of the air-dried material and find out the percentage of water soluble ash with reference to air dried sample taken.

Determination of sulphated ash value

Heat silica crucible to redness for 10 minutes; allow cooling in desiccators and weigh. Unless otherwise specified in the individual monograph, transfer to the crucible 1 gm of the substance under examination and weigh the crucible and the contents accurately. Ignite, gently at first, until the substance is thoroughly charred. Cool, moisten the residue with 1 ml of H_2SO_4 heat gently until the white fumes are no longer evolved and ignite at 800^{o} ± 25^{o}C until all black particles have disappeared. Conducted the ignition in a place protected from air current.

Extractive Values

This method is to determine the number of active constituents in each amount of medicinal plant material when extracted with solvent. It is employed for that material for which no chemical or biological assay.

Determination of alcohol soluble extractive value

5gm of air dried and coarsely powdered drug must be macerated with 100ml of ethanol (absolute alcohol) of the specified strength in a closed flask for 24 hours, shaking frequently during the first 6 hours and allowed to stand for 18 hours. Thereafter, filter rapidly taking precautions against loss of ethanol. Evaporate 25 ml of filtrate to dryness in a tarred flat-bottomed shallow dish, dry at 105^{o} C and weigh. The percentage of ethanol soluble extractive value with reference to the air-dried drug must be calculated **[19-21]**.

Determination of water-soluble extractive value

The water-soluble extractive value plays an important role for the evaluation of crude drugs. 5gm of the air dried and coarsely powdered drug must be macerated with 100 ml of water/chloroform water of the specified strength in a closed flask for 24 hours, shaking frequently during the first 6 hours and allowing for 18 hours. Thereafter, filter rapidly taking precautions against loss of water. Evaporate 25 ml of filtrate to dryness in a tarred flat-bottomed shallow dish, dry at 105^{o} C and weigh. The percentage of water-soluble extractive value with reference to the air-dried drug must be calculated.

Loss on Drying

Loss on drying is the loss of mass expressed as percentage w/w. It determines both water and volatile matter in the crude drug. It can be carried out either by heating at 100^{o} C -105^{o} C or in desiccators over phosphorus pent oxide under atmospheric or reduced pressure at room temperature for specified period **[22-24]**.

Procedure

About 2 gm of powdered drug was taken in a tarred porcelain dish which was previously dried at 105^{o} C in a hot air oven to constant weight and then weighed. Percentage of loss drying with reference to the air-dried substance was calculated **[25]**.

RESULTS AND DISCUSSION

Macro-Morphology of leaves

The macro-morphological characters of the leaves of *C.aurantium* and *C.sinensis* were studied and the results were documented as **Table 1**.

Micro-morphological characters of the leaves

The different shape of the cells, tissues and its inclusion, arrangements in sequence of leaf midrib, lamina and petiole have been studied extensively for better identification of the leaves of *C. aurantium* and *C. sinensis*. The results were documented and tabulated for comparison **(Table 2)**.

Preliminary phytochemical screenings

The preliminary phytochemical screenings of the leaf powder of Citrus aurantium and *C. sinensis* leaf revealed the presence of carbohydrates, proteins, and amino acids, saponins, steroids, glycosides,

flavonoids, terpenoids and phenolic compounds. It also shows that the alkaloids and tannins are absent in both drugs **(Table 3)**.

Physio-chemical parameters

The physio-chemical parameters were investigated and reported as total Ash value (6.3% w/w), Water soluble ash value (1.20% w/w), Acid insoluble ash value (2.60%), Sulphated ash value (2.90% w/w), Water soluble extractive value (12.75% w/w), Alcohol soluble extractive value (12.60% w/w), Loss on drying (8.90% w/w). The above studies were enabled to identify the plant material for future investigation and form an important aspect of drug studies **(Table 4, 5)**.

Table 1: Macro-morphological characters of leaves of *C.aurantium* and *C.sinensis* L. (Rutaceae)

S. No	Parameters	*C. aurantium*	*C. sinensis*
1.	Color	Whitish green	Green
2.	Odor	strongly scented	strong characteristic citrus
3.	Taste	Bitter and astringent	Bitter and astringent
4.	Texture	Hard and rough	Hard and papery
5.	Size	4-10 cm long	4-12 cm long and 6 cm wide
6.	Shape	Foliate, elliptic	elliptical, oblong to oval,
7.	Surface features	The shape of blades ranges from elliptical, bluntly serrated	The shape of blades ranges from elliptical, oblong to oval, bluntly toothed
8.	Leaf margin	Serrated margin	Crenulated margins
9.	Apex	Acuminate	Acute to obtuse
10.	Base	Symmetrical	Symmetrical
11.	Petiole	Winged	narrowly winged

Table 2: Micro-morphological studies of leaves of *C. aurantium* and *C. sinensis* L. (Rutaceae)

S. No	Parameters	*C. aurantium*	*C. sinensis*
1.	Anatomy of leaf	The midrib is biconvex with semicircular adaxial side and thick and prominent abaxial side. The midrib is 1mm thick in vertical plane. The adaxial part is 50µm in horizontal plane and the abaxial part is 900 µm wide. (fig.1.1)	Biconvex midrib and thick smooth lamina. The midrib consists of hemispherical adaxial part and thick convex abaxial part. The midrib is 1.15mm in vertical plane and 850µm in horizontal thick (adaxial part) and 1mm thick (abaxial part). (Fig 2.1).
2.	Epidermal layer	In midrib it is thin, and the cells are small and squarish; the cuticle is thick and smooths. Inner to the epidermis, there is wide cortical zone which is 250 µm wide. The cortex consists of angular compact parenchyma cells. The cells are smaller in the outer part and gradually increase in size towards interior (fig 1.2).	The epidermal layers have small rectangular cells with thick smooth cuticle. The mesophyll tissues consist of two layers of palisade on the upper part and about 10 layers of lobed spongy mesophyll cells which for reticulate partitions and wide air-chambers. (Fig 2.2)
3.	Secretary cavities	Located in the cortical regions which are circular and wide. The secretary cavity has two or more circles of epithelial cells which secrets the fluid into the cavity (fig 1.2).	Located in the cortical cells which are parenchymatous; the cells in the inner part of the cortex are small, circular, and darkly stained. The cells in the outer part are larger, circular, or elliptical in shape (fig.2.2).
4.	Sclereids	Encircling the vascular cylinder. It is thick continuous cylinder of brachy-sclereids; the sclereids are small and parenchyma like cells with very thick lignified cell walls and long canal like simple pits (fig.1.2).	The inner boundary of the cortex is demarcated by a thick cylinder of brachy sclereids; these sclereids are polyhedral in shape with thick lignified secondary wall and wide canal like simple pits (fig.2.2).
5.	Vascular cylinder	Deeply cup shaped vascular unit on the abaxial side and another smaller, plano-convex unit on the adaxial side. These vascular strands have several long, straight uniseriate xylem elements with xylem fibers located in between the narrow inter xylem spaces. The xylem elements are mostly circular, thick walled and lignified. The phloem consists of sieve elements, companion cells and phloem parenchyma. The phloem elements are small, thick walled and darkly stained (fig.1.2).	The vascular system of the midrib two independent segments- one is abaxial which is wide, and bowl shaped, another adaxial and planoconvex with semicircular adaxial part and flat abaxial part. The vascular segments are collateral with end arch protoxylem part. The metaxylem cells are circular, thick walled and they occur in parallel vertical files. Phloem occurs in thick continuous layer on the outer part of the xylem. Phloem includes small angular darkly stained elements (fig.2.2).
6.	Lamina	The lamina is bifacial with differentiation of the mesophyll tissues into adaxial palisade layer and abaxial spongy tissues. The spongy mesophyll is wide with wide air chambers separated by reticulate uniseriate partitions filaments. The lamina is 380 µm thick (fig 1.3).	The lamina is dorsiventral and smooth even surfaces. The epidermal layers have small rectangular cells with thick smooth cuticle. The mesophyll tissues consist of two layers of palisade on the upper part and about 10 layers of lobed spongy mesophyll cells which for reticulate partitions and wide air-chambers. The lamina is 320 µm thick (fig 2.3).
7.	Crystal distribution	The crystals are druses. They are spherical bodies with spiny surface. The druses are solitary in each cell (fig.1.4).	The crystals are druses, located either in the parenchyma cells located outside the sclerotic layer of external cells outside the scelreids; or the crystals are seen in the dilated epidermal cells and the crystals solitary in the cells (fig 2.4).
8.	Petiole	The petiole consists of epidermal layer, heterogeneous wide cortex and closed wide vascular cylinder. There is narrow, darkly stained central pith. The cortical region consists of small circular compact darkly stained cells. The inner region has larger circular less compact lightly stained cells. Xylem region of the petiole includes several radial lines of circular or elliptical vessels (fig.1.5).	The petiole consists of thin epidermal layer of small darkly stained cells. It consists of small circular parenchyma cells in the outer part and larger angular thin-walled cells in the inner part. The vascular strand is deeply curved with narrow opening towards the adaxial side. The strand consists of numerous, long, narrow xylem elements and xylem fibers. The xylem strands are ended with proto xylem elements being directed towards the center (fig.2.5).

Table 3: Results of the phytochemical constituents of leaf powder of *C. aurantium* L. and *C. sinensis.* L

PHYTOCONTITUENTS	*C. aurantium*	*C. sinensis*
Carbohydrates	+	+
Fixed oils and fats	_	_
Proteins and amino acids	+	+
Saponins	+	+
Steroids	+	+
Alkaloids	-	-
Glycosides	+	+
Flavonoids	+	+
Tannins	-	-
Gums and mucilage	-	-
Triterpenoids	+	+
Phenolic compounds	+	+

(+) Presence (-) Absence

Table 4: Determination of Ash values of leaf powder of *C. aurantium* L. and *C. sinensis.* L.

Ash values	*C. aurantium*	*C. sinensis*
Total Ash value	6.30	5.24
Water soluble ash	1.20	1.82
Acid insoluble ash	2.60	0.98
Sulphated ash	2.90	2.12

Table 5: Determination of extractive values and moisture content leaf of powder of *C. aurantium* L. and C. *sinensis.* L.

Extractive values	*C. aurantium*	*C. sinensis*
Alcohol soluble Extractive Value	12.60	14.15
Water soluble Extractive Value	12.75	16.42
Moisture content	8.90	5.50

Citrus aurantium

Legend for the figures

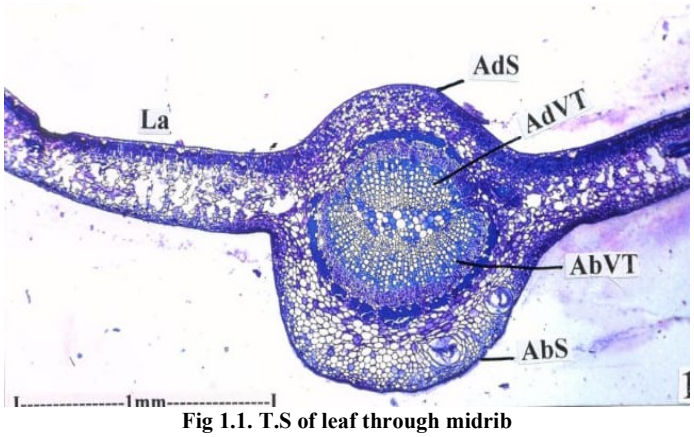

Fig 1.1. T.S of leaf through midrib

Ads- Adaxial side; Abs-Abaxial side; AdVT-Adaxial Vascular tissue; AbVT-Abaxial Vascular Tissue; La- Lamina

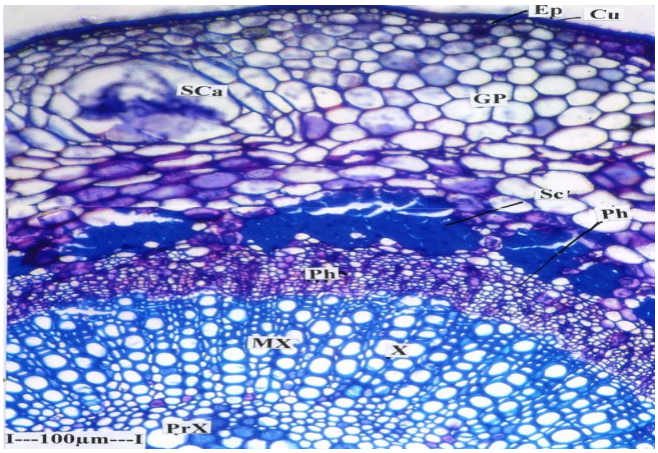

Fig 1.2. T.S of Midrib- a section enlarged
Cu-Cuticle; Ep-Epidermis; GP-Ground Parenchyma; MX-Meta Xylem; Ph-Phloem; PrX-Proto Xylem; Scl-Sclereids; Sca- Secretory cavity

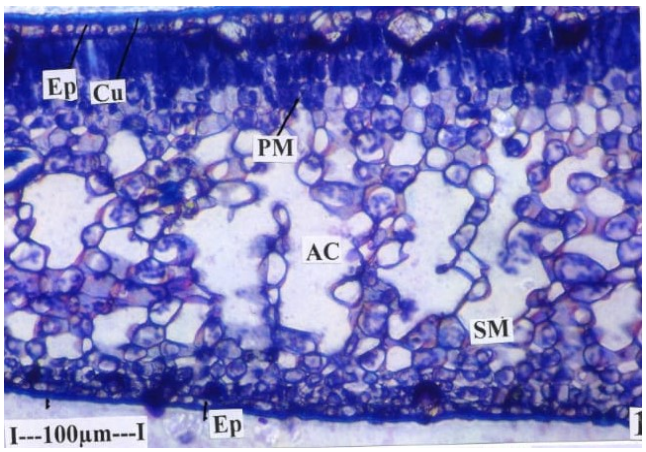

Fig 1.3. T.S of Lamina

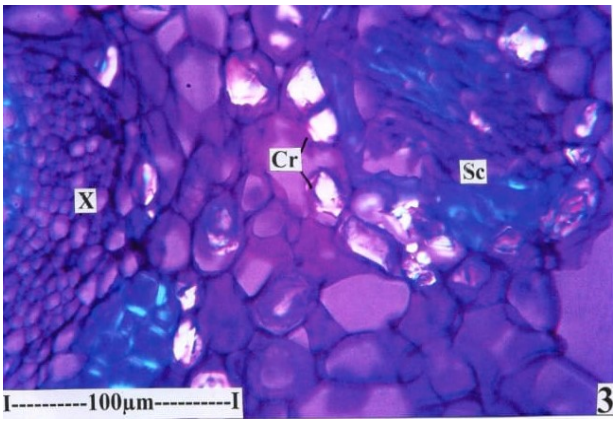

Fig 1.4. Calcium oxalate Druses in the parenchyma cells around the sclereids
AC- Air Chambers; Cu- Cuticle; Ep- Epidermis; Ads- Adaxial side; Abs- Abaxial side; Cr-Crystals; LM-Leaf Margin; PM-Palisade Mesophyll; SCa- Secretory Cavity; SM-Spongy Mesophyll; Sc- Sclereids; X-Xylem.

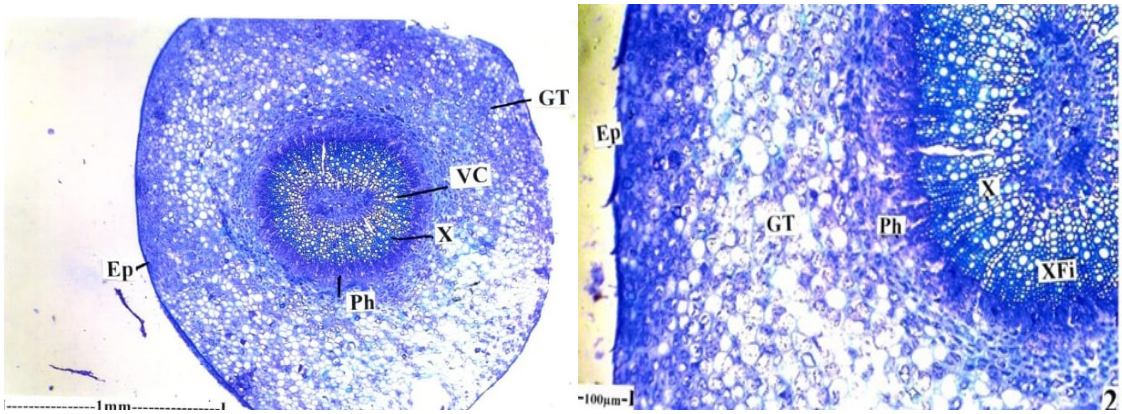

Fig 1.5. T.S of Petiole – outer view
Ep- Epidermis; GT-Ground Tissue; Ph-Phloem; VC-Vascular Cylinder; X-Xylem; XFi- Xylem Fibre

Citrus sinensis

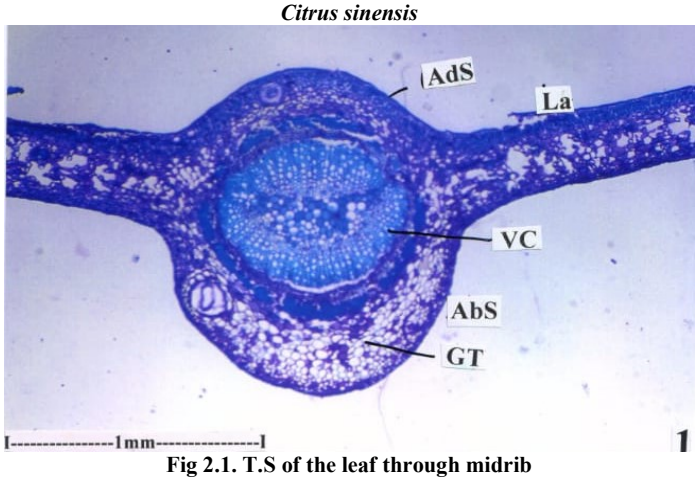

Fig 2.1. T.S of the leaf through midrib
Abs: Abaxial side; Ads: Adaxial side, GT: Ground Tissue; La: Lamina, Ph; Phloem, Se: Sclerenchyma; Sca; Secretary cavity, X: xylem

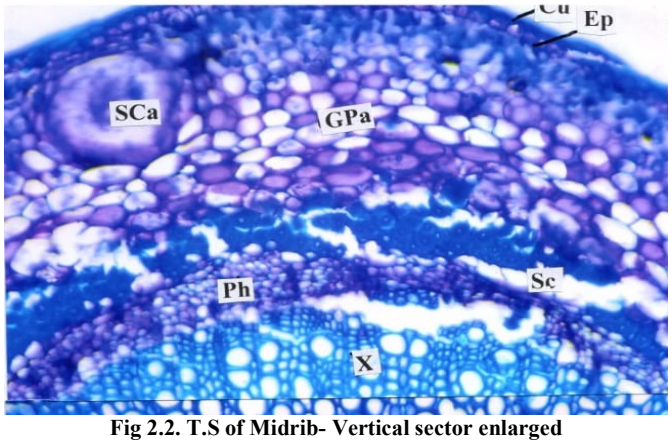

Fig 2.2. T.S of Midrib- Vertical sector enlarged
Cu; Cuticle, Ep; Epidermis; GPa; Ground Parenchyma, Ph; Phloem; Sc; Sclerenchyma, Sca; Secretary cavity, X: xylem.

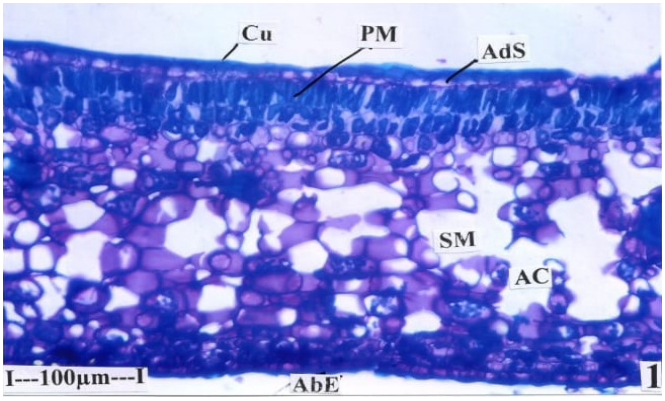

Fig 2.3. T.S of Lamina
AbE- Abaxial Epidermis; AdE- Adaxial Epidermis; Ae-Arenchymatous; Cu-Cuticle; LM-Leaf Margin; PM-Palisade Mesophyll; SM-Spongy Mesophyll; VS-Vascular Strand.

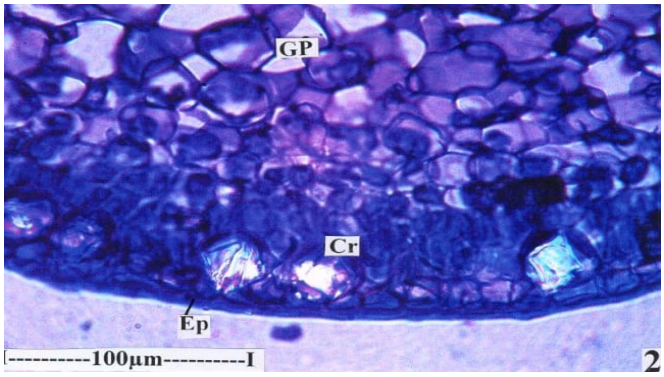

Fig 2.4. Localization of calcium oxalate crystals in the epidermal cells of the midrib.
Cr- Crystals; GP- Ground Parenchyma; Sc-Sclereids

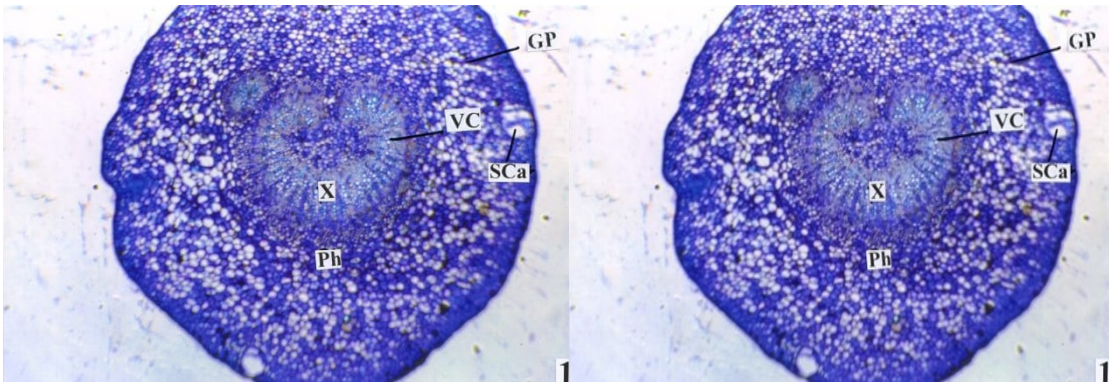

Fig 2.5. T.S of Petiole- entire view
AcV- Accessary vascular bundle; CPa- Cortical Parenchyma; GP- Ground Parenchyma; Ph-Phloem; SCa- Secretary Cavity; VC-Vascular Cylinder; X-Xylem.

CONCLUSION

The study lays foundation for pharmacognsotical standardization of two identically looking medicinal plants of Citrus species by comparing its micromorphological and physio-chemical parameters, which will help in identity, purity, and quality determination. The pharmacognostic analysis reported here which provides inclusive pharmacognostic profile of *Citrus aurantium* and *Citrus sinesis.* L. which will be helpful for correct

identification and authentication of the species for future studies.

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