PHARMACOGNOSTICAL, SEM AND EDAX PROFILE OF THE LEAVES OF *Citrus aurantium* L. (Rutaceae)

K.PERIYANAYAGAM*, S. DHANALAKSHMI, V.KARTHIKEYAN

Asst Reader, Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai 625 020, Tamil Nadu, India.

Email: kpn1960@yahoo.com

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**ABSTRACT**

**Objective**: To explore the micro morphology and SEM of *Citrus aurantium* L. (Rutaceae) leaves along with determination of trace elements by Energy dispersive X-ray analysis.

**Methods**: Macroscopy, microcopy including SEM, physicochemical analysis, preliminary phytochemical screening, EDAX and other WHO recommended parameters for standardizations were performed.

**Results**: Leaves (8-14cm × 4-5 cm) are dorsiventral, Foliate - elliptic, whitish green with serrate margin, acuminate apex and symmetrical base with winged petiole. Microscopic evaluation revealed the presence of cyclotytic stomata in lower epidermis and apotomatic upper epidermis, three layers of short palisade cells, wide circular secretory cavities, large double stranded vascular bundles, xylem vessels, phloem and fibers. SEM of midrib showed many folded appearance. No diagnostic feature and new kind of micro constituents not previously recognized and apparently simple structure which may be extremely complex was observed.

**Conclusion**: Microscopic analysis was informative and provides useful information in the botanical identification, standardization for purity & quality and immense value in authentication of the leaf. Elemental composition of the leaves useful in preparation of various herbal formulations with enriched minerals.

**Keywords**: *Citrus aurantium*, Rutaceae, Microscopical evaluation, Scanning Electron Microscopy (SEM), Energy Dispersive X-Ray (EDAX) analysis.

**INTRODUCTION**

*Citrus aurantium* L commonly called as bitter orange. The leaves of *Caurantium* really do not have any match as a cheap natural and easily available plant. It is traditionally known to be useful for the treatment of wide range of diseases like stomach ache, vomiting etc [1]. Leaf is traditionally used for emmenagoge[2], blood pressure[3], cough, cold, bronchitis[4], ear ache[5], dysentery, diarrhoea[6]. UI ailments[7], dysmenorrhea[8], influenza, insomnia[9], anti-inflammatory [10], headache [11], nervousness, weakness [12], hypoglycaemic, carminative [13], fever [14], sedative, digestive[15]. The leaves used as cytotoxic[16], antieast, antifungal and antibacterial[17]. Essential oil of the leaves used as antibacterial and antifungal[18], anxiolytic [19] and antiamoebic [20].

It was reported that fresh leaves contains: Flavone- Neodiosmin, Rhoifolin, Flavanone- Neohesperidin, Naringin[21]. In short, there is good level of traditional and experimental evidences to support various claims and advantages of this widely available plant. An investigation to explore its pharmacognostic examination is inevitable. Hence, in this work we report an attempt on microscopic evaluation, physicochemical determination and phytochemical screening for the standardization and quality assurance purposes of this cultivar.

**MATERIALS AND METHODS**

**Chemicals**

Formalin, acetic acid, ethyl alcohol, chloral hydrate, toludine blue, phloroglucinol, glyceral, hydrochloric acid and all other chemicals used in this study were of analytical grade.

**Plant collection and authentication**

The leaves of the healthy plant *Citrus aurantium* L. selected for our study was collected from Chinthamani, Villupuram District, Tamil Nadu, India during the month of January 2011 and was authenticated by Dr. P. Jayaraman, Director of Plant Anatomy Research Institute, Tambaram, Chennai, Tamil Nadu, India.

**Macroscopic analysis**

Macroscopic observation of the plant was done. The shape, size, surface characters, texture, color, odour, taste etc was noted [22].

**Microscopic analysis**

Transverse section midrib region of fresh leaf pieces were cut and fixed in FAA and then dehydrated by employing graded series of ethyl alcohol and tertiary butyl alcohol [23]. Sections were taken using Microtome. Permanent mount was prepared using saffranin fast green double staining technique [24]. In order to supplement the descriptive part the photomicrographs in different magnifications of all necessary cells and tissues were taken with NIKON Coolpix 8400 digital camera and LabphotZ microscopic unit.

**Powder microscopy**

Coarse powder of the leaf was used to study the microscopical characters of the leaf powder [25, 26].

**Physicochemical analysis**

Total ash, acid insoluble ash, water soluble ash, loss on drying, extractive values and leaf constants such as vein islet numbers, vein terminal number, stomatal number and stomatal index, palisade ratio were determined [27].
Preliminary phytochemical screening

Preliminary phytochemical screening was carried out to find out the presence of various phytoconstituents using standard procedure [28].

Scanning electron microscopic study

Scanning electron microscopy is a complementary technique and importance in pharmacognostic evaluation [29].

SEM sample preparation

Sample for SEM analysis were mounted on the specimen stub using carbon adhesive sheet. Small sample were mounted with 1sq. cm glass slide And kept in carbon adhesive sheet. Samples were coated with gold to a thickness of 100 AO using Hitachi vacuum evaporator. Coated sample were analyzed in a Hitachi Scanning electron Microscope 3000 H model.

Elemental analysis by EDAX

EDAX is a non destructive technique and can be used for multiple sampling in various parts of the plant and can also provide information from an area of fewer nanometers. This is very useful to characterize the crystals and other inclusions like trace elements [30].

RESULTS

Macroscopy

*C. aurantium* is a tree with greenish white, glabrous shoots. Leaves (8-14cm × 4-5 cm), dorsiventral, foliate – elliptic, serrated margin, and whitish green in colour, acuminate apex and symmetrical base with winged Petiole. Flower bisexual, pure white. Stamens 20-30. Fruit globose, generally oblate, not mamillate, usually orange-coloured; rind loose or adherent; Pulp sweet, yellow, rarely red (Fig 1).

Microscopy of the leaf

Transverse section (T.S) of the leaves through the midrib showed the following tissue systems.

Shape: Leaves are dorsiventral with prominent midrib, Foliate to elliptic, acuminate. In transactional view it is Prominent elevated round in shape, 630μm thick, 300µm wide adaxial side and comparatively less thick abaxial side. (Fig 2)

Figure 1: Habit of *C. aurantium* L.

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Figure 2: Dorsal and ventral view of the leaves of *C. aurantium*

Epidermis: Upper epidermal cells Squarish thick walled with smooth cuticle. Polygonal in surface view with thick straight wall. Apostomatic. Lower epidermal cells were thick with papillate surface due to cuticular outgrowth slightly wavy larger cells.

Mesophyll: The mesophyll is differentiated into adaxial zone of three layers of short palisade cells and wider abaxial zone of compact layers of spongy parenchyma with wide air chambers in the middle part vascular strands of lateral veins are present. Wide circular secretory cavities surrounded by fairly thick spindle shaped epithelial cells (200 µm diameter) with amorphous inclusion. Exclusively abundant prismatic calcium oxalate crystals. The distribution pattern is characteristic. They are located in sub epidermal layers of adaxial epidermis. The cell bearing crystals are wide circular filled with mucilage and called as crystal Idioblast. Vascular system is large and double stranded. Several short, compact, parallel rows of xylem (both vessel and fibre).The vessels are angular to circular and thick walled 1 µm wide. Phloem present as thick is beneath the abaxial bundle and outside the xylem of abaxial strand. Ground tissue made up large thin walled compact parenchyma cells (Fig 3).

Figure 3: T.S of the Leaf of *C. aurantium* through Midrib (4×)

Petiole: Transverse section of petiole is round. Flat on the abaxial side and semicircular on the adaxial side, 1.7mm thick, 1.8mm wide. Epidermis made by thin cubicle cells. Vascular strands were closed hollow cylinder 1mm in diameter. Xylem occurs as long unisertate parallel xylem elements containing both vessels and fibres. Phloem occurs as continuous like thick sheath all around the xylem cylinder containing phloem elements and phloem parenchyma. Calcium oxalate druses prismatic crystals present in normal cells not modified into Idioblast unlike in Lamina. Ground tissue parenchymatous in nature. Secretary cavities especially more in adaxial part and similar as in lamina (Fig 4).
3.3 Powder microscopy: The analysis of the dried powder of the leaf showed parenchyma cells; epidermal cells with cyclocytic stomata, secretory cavities, crystal idioblast, prismatic cells druses, collenchyma, fibres, xylem, phloem were noticed (Fig 5).

Figure 4: T.S of the Petiole

Figure 5 Powder Microscopy of C. aurantium Leaves

Physicochemical analysis
Physicochemical parameters were found as follows: total ash 9.82% w/w, acid insoluble ash 1.8, water soluble ash 5.4 % w/w, ethanol soluble extractive value 0.68 % w/w, water soluble extractive value 7.58 % w/w, petroleum ether soluble extractive 0.48%, loss on drying 1.9% w/w and foreign organic matter was nil. Leaf constants were as follows vein islet number 3.38, vein termination number 6, stomatal number (lower epidermis) 67.75, stomatal index (lower epidermis) 16.42 and palisade number 3.25.

Preliminary phytochemical screening
Preliminary phytochemical screening showed the presence of flavonoids, terpenoids, sterols, tannin, volatile oil, saponins, proteins and amino acids, carbohydrates, reducing sugars, and absence of alkaloids, cyanogenetic glycosides, anthroquinone glycosides, cardiac glycosides, mucilage and fixed oil.

SEM of leaf
Scanning Electron Microscopy of midrib showed many folded appearance. No diagnostic feature and new kind of micro constituents not previously recognized and apparently simple structure which may be extremely complex was observed (Fig 6).

Figure 6: SEM Analysis of Leaf

EDAX analysis of leaf
Energy dispersive X-ray analysis (EDAX) showed the presence of minerals Calcium (0.42%), Potassium (0.72%), Magnesium (0.09%), and Sodium (0.09%).

DISCUSSION
Sensory evaluation plays a key role in determining the suitability or denunciation of a crude drug. Organoleptic testing of a crude drug is mainly for qualitative evaluation based on the observation of morphological and sensory profile [30]. In this report, various morphological, microscopic, physicochemical standards have been developed. Hence we have undertaken this study to serve as a tool for developing standards for identification, quality and purity of C. aurantium leaves.

Adulteration and misidentification of crude drugs can cause serious health problems to consumers and legal problems for the pharmaceutical industries. The introduction and implementation of new Good Manufacturing Practices (GMP) in quality control of raw materials, intermediates and finished products of botanical origin. The initial step in quality control of medicinal plants is ensuring the authenticity of the desired species for the intended use. It can be conducted via a variety of techniques, namely macro and microscopic identification and chemical analysis especially description of microscopic botanical aspects to determine definitively the proper species of plant material while it is still in its non extracted form. The observation of cellular level morphology or anatomy is a major aid for the authentication of drugs. These characters are especially important for identification of powdered drugs, because in these cases most of the morphological diagnostic features are lost [31]. Microscopic evaluation is one of the simplest and cheapest methods for the correct identification of the source of the materials [32]. The macroscopic and organoleptic characters of the leaf can serve as diagnostic parameters [33]. Presence of cyclocytic stomata in the lower epidermis, where the stoma was encircled by three inner whorls, 5 or 6 outer whorls of subsidiary cells. 3 or 4 layers of collenchyma were present beneath both the epidermis. Large double stranded vascular bundles were seen.
A characteristic three layers of short palisade cells and wide abaxial zone of compact layers of spongy parenchyma with wide air chambers in the middle part were observed. Wide circular secretory cavities surrounded by fairly thick spindle shaped epithelial cells with amorphous inclusion were present. A characteristic exclusively abundant prismatic calcium oxalate crystals distributed in sub-epidermal layers of adaxial epidermis were noticed. Polygonal vein islets with thick vein boundaries and distinct dendroid vein termination were present. The outline of petiole was almost round. Flat on the abaxial side and semicircular on the abaxial side. Secretary cavities are more in adaxial side. Calcium oxalate druses and prismatic crystals were present in normal cells not modified into idioblast unlike in lamina was characteristic feature. The scanning electron microscopy study showed the above structures in 3D view.

The physical constant evaluation of the drug is an important parameter in detecting adulteration or improper handling of drugs. The ash values are particularly important to find out the presence or absence of foreign inorganic matter such as metallic salts and or silica (earthy matter). Acid insoluble ash provides information about non-physiological ash produced due do adherence of inorganic dirt, dust to the crude drug. Increased acid insoluble ash indicates adulteration due do dirt, sand or soil [3,4]. The extractive values are primarily useful for the determination of exhausted or adulterated drug and helpful in the detection of adulteration [35]. Phytochemical evaluation and molecular characterization of plants is an important task in medicinal botany and drug discovery [36]. Preliminary phytochemical screening showed the presence of sterols, flavonoids, terpenoids, saponins, volatile oil, protein and aminoaacids, reducing sugars, carbohydrates, and absence of sterols, flavonoids, terpenoids, saponins, volatile oil, protein and aminoacids, reducing sugars, carbohydrates, and absence of alkaloids, fixed oil, mucilage and glycosides. It is also used often as a diagnostic feature to avoid misleading by over simplified descriptions and one may find new kinds of microstructures not previously recognised and apparently simple structures may be extremely complex. SEM plays a vital role when a specimen needs to be satisfactorily defined in terms of characters. For most biological materials, maximum information is obtained by employing light and electron microscopy jointly and an attempt was made by applying SEM [37]. Scanning Electron Microscopy of midrib showed many folded appearance. No diagnostic feature and new kind of micro constituents not previously recognized and apparently simple structure which may be extremely complex was observed. Trace elements are considered the "inorganic switches" in various medicinal systems. This concept has gained ground in Ayurveda and the traditional Indian medicinal systems [38]. Mineral contents of various medicinal plants correlated with their therapeutic action by numerous studies [39,40]. Energy dispersive X-ray analysis (EDAX) showed the presence of minerals Calcium (0.42%), Potassium (0.02%), Magnesium (0.09%), and Sodium (0.09%).

CONCLUSION

The present work was undertaken with a view to lay down standards which could be useful to detect the authenticity of this medicinally useful plant Citrus aurantium leaves to control various diseases. Microscopical evaluation, XRF and physicochemical standards and preliminary phytochemical reports can be useful to substantiate and authenticate drug.

Conflict of interest statement

We declare that we have no conflict of interest.

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