INNOVARE JOURNAL OF HEALTH SCIENCES

Vol 1, Issue 2 , 2013

Research Article

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

Received: 31July2013 Revised and Accepted: 31August 2013

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, microscopy 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 cyclocytic stomata in lower epidermis and apostomatic upper epidermis, three layers of short palisade cells, wide circular secretary 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.
Identification of inorganic minerals of the leaves of *C.aurantium* by EDAX showed the presence of minerals Calcium (0.42%), Potassium (0.72%), Magnesium (0.09%) and Sodium (0.09%).
Vein islet numbers, vein termination numbers, stomatal number, stomatal index and other physico chemical tests like ash values, loss on drying, extractive values were determined. Preliminary phytochemical screening showed the presence of sterols, tannins, proteins and aminoacids, flavonoids, volatile oil, terpenoids, saponin, carbohydrates and absence of alkaloids, mucilage, glycosides and fixed oil.
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 *C.aurantium* 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 panel of diseases like stomach ache, vomiting etc [1]. Leaf is traditionally used for emmemagogue[2], blood pressure[3], cough, cold, bronchitis[4], ear ache[5], dysentery, diarrhea[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], antiyeast, 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, glycerin, 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 Labphot2 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).

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

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 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 mesophill 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 secretary 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).

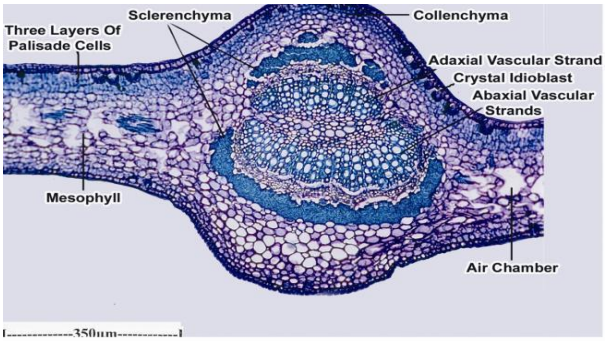

Figure 3: T.S of the Leaf of *C. aurantium* through Midrib (4×)

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 uniserriate 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).

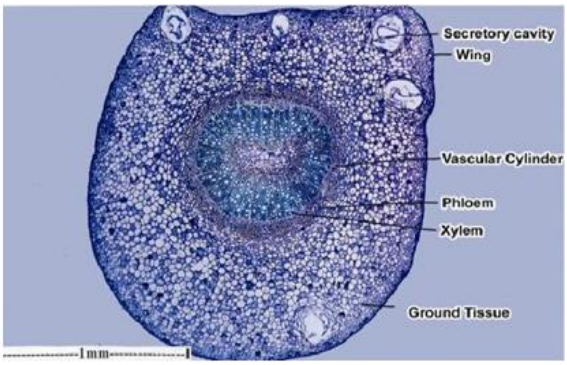

Figure 4: T.S of the Petiole

3.3 Powder microscopy: The analysis of the dried powder of the leaf showed parenchyma cells; epidermal cells with cyclocytic stomata, secretary cavities, crystal idioblast, prismatic cells druses, collenchyma, fibes, xylem, phloem were noticed (Fig 5).

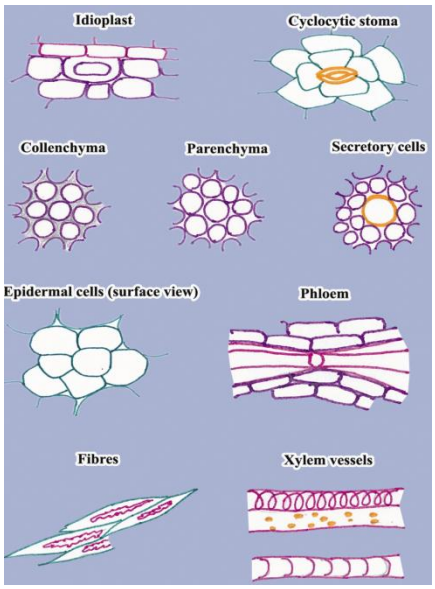

Figure: 5 Powder Microscopy of *C. aurantium* Leaves

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).

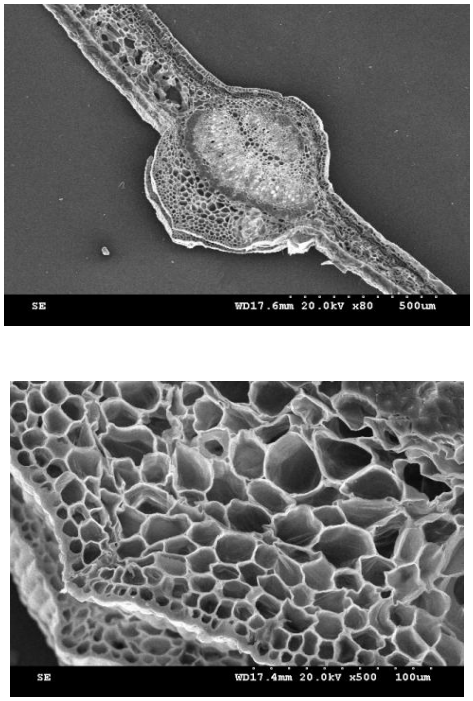

Figure 6: SEM Analysis of Leaf

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, microscopical, 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 secretary 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. Secretory 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 [34]. 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 aminoacids, reducing sugars, carbohydrates, and absence of alkaloids, fixed oil, mucilage and glycosides. It is also used often as 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 (0.72%), 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.

ACKNOWLEDGEMENT

The author thanking for all helping hands particularly Dr. Stephen, Department of Botany, American college, Madurai for plant authentification and Dr. P. Jayaraman, Director of Plant Anatomy Research Institute, Tambaram, Chennai for Microsopical studies.

REFERENCES

1. Anonymous. Wealth of India Raw materials. Ca-Ci, Vol III. National Institute of Science Communication and Information Resources (NISCAIR), CSIR: New Delhi; 2005.
2. Morton JF. A survey of medicinal plants of curacao. Econ Bot1968; 22: 87-92.
3. Comerford SC. Medicinal plants of two mayan healers from San andres, Peten, Guatemala. Econ Bot1996; 50(3): 327-36.
4. Whistler WA. Traditional and herbal medicine in the Cook Islands. J Ethno pharmacol1985; 13(3): 239-80.
5. Gurib-Fakim A, Sweraj MD, Gueho J, Dullo E. Medicinal plants of Rodrigues. Int J Pharmocog1996; 34(1): 2-14.
6. Hedberg I, Hedberg O, Madati PJ, Mshigeni KE, Mshiu EN, Samuwlssom G. Inventory of plants used in traditional mediine in Tanzania. Part III. Plants of the families' Papilionaceae-Vitaceae. J Ethno Pharmacol1983; 9(2/3): 237-60.
7. Coe FG, Anderson GJ. Screening of medicinal plants used by the Garifuna of Eastern Nicaragua for Bioactive compounds. J Ethno Pharmacol1996; 53: 29-50.
8. Browner CH. Plants used for reproductive health in Oaxaca, Mexico. Econ Bot1985; 39(4): 482-504.
9. Darias V, Bravo L, Barquin E, Herrera DM, Fraile C. Contribution to the ethnopharmacological study of the Canary Islands. J Ethno Pharmacol 1986; 15(2): 169-93.
10. Cha S. Potential anticancer medicinal plants. A statistical evaluation of their frequencies of appearance in oriental medicine formulation. Korean J Pharmacog1977; 8: 1.
11. Weniger B, Rouzier M, Daguilh R, Henrys D, Henrys JH, Anton R. Popular medicine of the central plateau of Haiti. Ethnopharmacological Inventory. J Ethno Pharmacol1986; 17(1): 13-30.
12. Glron LM, Freire V, Alonzo A, Carceres A. Ethanobotanical survey of the medicinal flora used by the Caribs of Guatemala. J Ethno Pharmacol 1991; 34 (2/3): 173-87.
13. Darias V, Abdala S, Martin D, Ramos F. Hypoglycaemic plants from the Canary Islands. Phytother Res Suppl. 1996; 10: S3-S5.
14. Barrett B. Medicinal plants of Nicaragua's Atlantic coast. Econ Bot1994; 48 (1): 8-20.
15. De-feo V, Senatore F. Medicinal plants and phytotheraphy in the Amalfitan coast, Salerno province, Campania, Southern Italy. J Ethano Pharmacol1993; 39(1): 39-51.
16. May G, Willuhn G. Anti viral activity of aqueous extracts from medicinal plants in tissue cultures. Arzneim-Forscb1978; 28(1): 1-7.
17. Verpoorte R, Dihal PP. Medicinal plants of Surinam IV. Antimicrobial activity of some medicinal plants. J Ethno Pharmacol1987; 21(3): 315-18.
18. EL-Keltawi NEM, Megalla Se, Ross SA. Antimicrobial activity of some Egyptian aromatic plants. Herbo Pol 1980; 26(4): 245-50.
19. CarvalhoFreitas MIR, Costa M. Anxiolytic and sedative effects of extracts and essential oil from *Citrus aurantium* L. Biol Pharm Bull 2002; 25(12): 1629-33.
20. De blasi V, Debrot S, Menoud PA, Gendre L. Schowing J. Amoebicidal effect of essential oils *in vitro*. J Toxicol ClinExp 1990; 10(6): 361-73.
21. Rio JAD, Benavente O, Castillo J, Borrego F. Neodiosmin, a flavone glycoside of *Citrus aurantium*. Phytochemistry 1992; 31(2): 723-24.
22. Kokate CK, Gokhale SB, Purohit AP. Pharmacognosy. 46th Edn. NiraliPrakashan: New Delhi; 2010. A.1-6.
23. Asokan J. Botanical microtechnique principles and practice.1st edn. Plant anatomy research centre: Chennai; 2009.

24. Kunle, FolashadeO, Egharevba, Omoregie H, Ahamadu, Ochogu P. Standardization of herbal medicines-A Review. Int J Biodiver Conser. 2012; 4(3):101-12.
25. Evan WC. Trease and Evans Pharmacognosy. 16th edn. Elsevier: London Saunders; 2009. P563-70.
26. WHO. Quality control methods for medicinal plant materials. World Health Organisation: Geneva; 1998.
27. Mukherjee PK. Quality control of Herbal drugs- An approach to evaluation of botanicals 1st edn. Business Horizon: New Delhi; 2012.
28. Vadlapudi V, Kaladhar DSVGK. Phytochemical evaluation and molecular characterization of some important medicinal plants. Asian Pac J Trop Diseases 2012; 2(1): S26-32.
29. Yashvanth S. Rani SS. Rao AS. Madhavendra SS. Anatomical exploration of *Leucas aspera* (Willd) links a medicinal herb and its pharmacognostic relevance. J Pharma Res 2011; 4(12):4777-79.
30. Pradhan AN, Agrahari AK, Meher A, Mishra MN, Elemental analysis by energy Dispersive x-ray spectroscopy (EDX) of *Capparis zeylanica* Linn. Plant. J. Pharma Res 2010; 3(4): 669-70.
31. Chan YY, Lo SCL. Analysis of Ling Zhi (*Gonoderma lucidum*) using dynamic reaction cell ICP-MS and ICP-AES. J. Anal. Atomic Spectrometry 2003; 18: 146-50.
32. Serrano R, Silva G, Silva O. Application of light and scanning electron microscopy in the identification of herbal medicines. In: Mendez vilas A, Diaz J. (eds). Microscopy: Science, Technology, Application and Education. Vol.1, Sapin, Formatex Research Centre; 2010, p182-90.
33. Singh S, Machawal L, Chauhan MG. Pharmacognostic study of male leaves of *Trichosanthes dioica* Roxb. with special emphasis on microscopic technique. J Pharmacognosy Phytother 2010; 2(5): 71-75.
34. Nayak BS, Patel KN. Pharmacognostic studies of the *Jatropha curcas* leaves. Int J Pharm Tech Res 2010; 2(1): 140-43.
35. Pimple BP, Patel AN, Kadam PV, Patil MJ. Microscopic evaluation and physicochemical analysis of *Origanum majorana* Linn leaves. Asian Pac J Trop Diseases 2012; 2(2): S897-903.
36. Patel S, Zaveri M. Pharmacognostic study of the root of *Justicia gendarussa* Burm. J Trad Med 2011; 6(2): 61-72.
37. SandipkumarBhat P, SaurabhPandya S. SEM and TEM study of bark of *Listea chinensis* Lam. J Phar Res 2012; 5(2):1260-63.
38. Under Wood EJ. Trace metals in human and animal health. J. Hum. Nutr.1981; 53:37-48.
39. Sahito SR, Memon MA, Kazi, TG, Kazi GH, Jakhrani MA, HaqueQU, Shar GQ. Evaluation of mineral contents in medicinal plant *Azadirachta indica* (Neem). J. Chem. Soc. Pak. 2003; 25(2):139-43.
40. Pirzada AJ, Iqbal P, Shaik W, Kazi TG, Ghani KU. Studies on the elemental Composition and antifungal activity of medicinal plant *Lippia nodiflora* L. Against skin fungi. J. Pak Assoc. Dermat 2005; 15: 113-18.