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AUTHENTICATION OF HERBAL MEDICINE NEEM (*AZADIRACHTA INDICA* A.JUSS.) BY USING TAXONOMIC AND PHARMACOGNOSTIC TECHNIQUES

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Abstract

The quality assurance of Neem (*Azadirachta indica* A.Juss.), a traditional herbal drug of global importance used for the treatment of different ailments, was studied. At global, regional, national and local levels, the end users of this drug face the problem of adulteration. Two different species are commercially marketed in the Indo-Pak Subcontinent under the same trade name of Neem. One is *Azadirachta indica* and the other is *Melia azedarach* L., both belonging to Meliaceae. In this study, a commercially available drug sample of Neem was authenticated by using basic and advanced Taxonomic and pharmacognostic analysis. Authentication, quality and standardization of this drug were achieved using morphology, organoleptography, pharmacogonistic markers, UV and IR analyses, SEM of pollen and anatomical investigations. This study contributes towards the global recognition and international acceptance of Neem as an herbal drug.

Introduction

Medicinal plants are of great value in the treatment and cure of diseases. Over the years, scientific research has expanded our knowledge of medicinal plants and new drugs. In the Western world, as people are becoming aware of the potency and side effects of synthetic drugs, there is an increasing interest in plant-based medications. The future development of the pharmacognostic analysis of herbal drugs is largely dependent upon reliable methodologies for correct identification, standardization and quality assurance of herbal drugs. Describing herbal drugs in a systematic manner is based on multiple approaches of pharmacognostic, taxonomic and chemical analysis, including documentation of their biological and geographical source, cultivation, collection, processing, morphological, microscopic and chemical characters.

The increase in demand for herbal medicines may lead to indiscriminant and unscientific collection, misidentification, and adulteration without any standards for quality of the material. According to Handa (2004), the majority of medicinal plants used by the herbal drug industry and local communities come from wild collection. The raw material used by the drug industry and communities in large cities, towns and regions is generally procured through market channels and is sometimes found adulterated.

Problems associated with incorrect identification, substitution and adulteration of plant material, sometimes accidental, sometimes deliberate, had not gone unnoticed by those who use the plant materials. In the Indo-Pak Subcontinent the herbal industries and local communities generally face the problems of adulteration and substitution at a raw material stage. Ahmad *et al*., (2010) and Khan *et al*., (2011) observed that in herbal markets of India and Pakistan, sometimes entirely different taxa are being sold under the same local or common name (e.g. under the trade name of neem/nim, two or more different taxa are being sold at herbal shops of the Indo-Pak Subcontinent). In this study, the herbal drug Neem (*Azadirachta indica*) was selected as a case study for methods to authenticate and distinguish it from its adulterant *Melia azedarach*, based on taxonomic and pharmacognostic analyses. The aim of the study was to characterize and accurately identify Neem, a commonly traded and used herbal drug among the communities of the Indo-Pak Subcontinent.

Materials and Methods

Morphological study: Morphological observations were made from living plants collected during seven field trips and procured from 18 herbal shops. Morphological studies (root, leaf, stem and flowers) were also based on examinations of 20 herbarium specimens available at ISL-QAU Herbarium, Islamabad. Further information from taxonomic and floristic sources provided confirmation of morphological characteristics (Hooker, 1875; Tutin & Heywood, 1972; Nasir & Ali, 1974; 1975). Morphological examinations conducted using a binocular stereo zoom light microscope (Model SZF Kyowa, Japan, with eye piece WF 10 x 10/20). Assessment of floral morphology was aided by reconstitution of dried flowers in hot water with detergent. All the field images presented were taken by the author using a Sony Digital Camera (DSC-W50).

Organoleptography: The organoleptic analysis was based on field collection and samples of crude herbal parts procured from herbal shops. The crude herbal samples were cleaned, washed and dried in shade. Physical characters (color, smell and taste) of the samples were studied. The crude herbal samples were numbered and preserved in glass bottles and zipped plastic envelopes. The macroscopic features of the herbal parts were studied with a Zeiss binocular light microscope.

Palynological study (LM & SEM): A palynological study of *Azadirachta indica* and its adulterant *Melia azedarach* was conducted using light microscopy (LM) and scanning electron microscopy (SEM). The pollen samples studied were obtained from fresh collections. Fully matured anthers were removed from specimens and prepared by the standard procedure of acetolysis (Erdtman, 1960), after which they were mounted in glycerin jelly and sealed with paraffin wax for light microscopy. The glycerin jelly was prepared according to modified method of Zafar *et al*. (2011). Measurements and morphological observations of pollen grains were performed using a minimum of 15 grains for each species.

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For scanning electron microscopy (SEM), the flowers were opened under a binocular dissecting microscope (Meiji MX5200H) using a dissecting needle. Pollen grains were fixed to aluminum stubs with double sided cellophane tape, air dried at room temperature and coated with a very thin layer of gold (JFC-1100). The specimens were examined using a scanning electron microscope (JEOL-JSM 5910), at 2000x, 5000x and 10000x magnification. The terminology used for sculpturing is based on the work by Erdtman (1960), Barthlott (1984) and Ronald (2000).

Anatomical study (LM & SEM): For epidermal preparations, leaf samples of 1 to 3 cm were cut from the mid portion of mature foliage leaves. Shultze's method, with modifications, was used (Subrahmanyam, 1996; Zafar *et al*., 2011). The peelings of leaves were washed with distilled water for 2-3 minutes. The leaf blades were placed with the adaxial side upward and then scraped gently with a sharp razor. The same procedure was followed to prepare the abaxial side but the leaf was placed with the abaxial surface upward. The abaxial and adaxial epidermal peelings were kept in lactic acid for few minutes in order to remove mesophyllous tissues and extra chlorophyll. Then the peelings were placed on clean glass slides with 1-2 drops of 88% lactic acid, covered with cover slips and fixed with paraffin wax. Prepared slides were observed under a Meiji light microscope. The microphotographs of adaxial and abaxial surface were taken with a Leica light microscope fitted with a CCD camera (DM – 1000). The same procedure was adopted to peal of the leaves from adaxial and abaxial surfaces for SEM study. The peelings were dried at room temperature and then affixed to stubs with double sided tape and coated with the same manner as the pollen. Descriptions of foliar epidermal features follow the terminology of Prat (1932) and Metcalfe (1960).

Pharmacognostic studies: Different pharmacognostic tests, i.e., fluorescence and solubility test (cold and hot) (Tables 2, 3, 4 & 5), were carried out for crude herbal parts of *Azadirachta indica* and *Melia azedarch* in order to distinguish which of the two plants is found in the herbal drug. For cold method 1 gm of powdered drug was mixed in 5 ml of solvent at room temperature (10–15^{o}C), while for hot method the same solution was slightly heat on burner in a test tube. The methods of Harborne (1973), Trease & Evans (1989) and Sofowara (1993) were followed. All the chemicals and solvents used for the different studies were of HPLC grade.

For solubility and florescence analysis standard procedures were adopted (Afaq *et al*., 1998; Abid *et al*., 2005). The fresh collected herbal drug (leaf material) of the medicinal plants was dried in outdoor shade for about 10-15 days and made into a powder by using an electric grinder. Crude herbal drug samples (leaf material) procured from markets were also made into powder. This coarse powder was sieved into a fine powder by using a No. 10 sieve. The fine powder was used for the extraction and determination of various physico-chemical properties. One gram of the powdered drug was mixed separately with 5 ml of 19 different solvents (Table 2); samples prepared by the hot method were boiled in test tubes. Crude herbal parts, powdered drugs and the extracts were studied under visible light, UV (long & short wavelength) and IR lights following the procedure of Ahmad *et al*., (2010). For color analysis, a paint chip card from Indigo Company (Pakistan) was used for comparison.

Results and Discussion

Azadirachta indica A.Juss. (family: Meliaceae, common name: Neem) is well known in the Indo-Pak Subcontinent and neighboring countries as one of the most versatile medicinal plant used by humans to combat diseases. Neem, an evergreen tree, is indigenous to South Asia. In African countries, it is abundant in a tropical belt from Somalia to Nigeria. Introduced first into Fiji, it has spread to many islands in the South Pacific, and from Trinidad it has spread to other islands in the West Indies and many countries in Central and South America. It has been introduced in Florida and Southern California. Large-scale plantations have been planted in Malaysia and the Philippines. Thousands of trees have been planted outside Makkah for affording shade to pilgrims. Thus, Neem has spread too many frost-free parts of the world (Anon., 1985; Ahmad, 1988; Stone, 1992).

From very ancient times, various parts of the tree have been used as traditional medicine against various human ailments. Neem has been extensively used in Unani, Ayurveda and homeopathic medicine and has become a cynosure of modern medicine. In Sanskrit the name Arishta, meaning "the cure all", is given to Neem, whereas Ayurveda regards the tree as Sarva Rogha Nirvarini, meaning "cure all ailments". The importance of the tree has also been recognized by the US National Academy of Sciences by titling Neem as "The tree for solving global problems (Kraus, 1995; Singh *et al*., 1996).

In the Indo-Pak Subcontinent, the Greco-Arab and Ayurveda systems of medicine are considered the oldest systems of medicine. Plants are generally referred to and published in folk literature by their vernacular or common names rather than botanical names. Due to this confusion, medicinal plant material is often adulterated with closely related species. The main difficulty in fixing the botanical identity of medicinal plants in ancient Unani literature and traditional systems arises due to the local name(s) of these plants, with local name(s) applied to more than one plant species. The actual botanical species of a large number of folk medicines found to be therapeutically effective in indigenous systems are still unknown or doubtful. Many workers (Ahmad 1988; Afaq, 1998; Girach *et al*., 1998) have stressed the need for authentic botanical identification of herbal plants used in the Unani system of medicine, in order to maintain herbal drug efficacy.

For instance, *Azadirachta indica* has the local and trade name of Neem and is found throughout the Indo-Pak Subcontinent, but its distribution is not uniform in the region and it is restricted to localized areas. The vernacular name or trade name Neem has been erroneously used for the closely related species *Melia azedarach* by many herbalists, local communities and herb sellers (Khan *et al*., 2000). Due to their morphological similarities, *Azadirachta indica* is commonly adulterated with *Melia azedarach* at herbal shops. The problems of adulteration and nomenclatural controversy in this case may lead to misuse of this plant for specific diseases. In this study, a systematic approach

was adopted to authenticate samples of *Azadirachta indica* as the herbal medicine Neem by using taxonomic and pharmacognostic analyses. Taxonomic and pharmacognostic analyses of *Azadirachta indica* and *Melia azedarach* are presented in Tables 1, 2, 3, 4 and 5. Taxonomic characterization includes morphological, organoleptic, palynological and anatomical features. Morphologically, *Azadirachta indica* is an evergreen medium sized tree up to 20 m tall, with light green opposite pinnate leaves (Fig. A1), while *Melia azedarach* is a deciduous tree up to 45 m tall, with dark green alternate and bipinnate leaves (Fig. A2).

Azadirachta indica

Fig. A1. *Azadirachta indica* (Aerial Branch).

Melia azedarach

Fig. A2. *Melia azedarach* (Aerial Branch).

Fig. B1. Dried aerial parts.

Fig. B2. Dried aerial parts.

Fig. C1. Dried aerial parts under UV & IR.

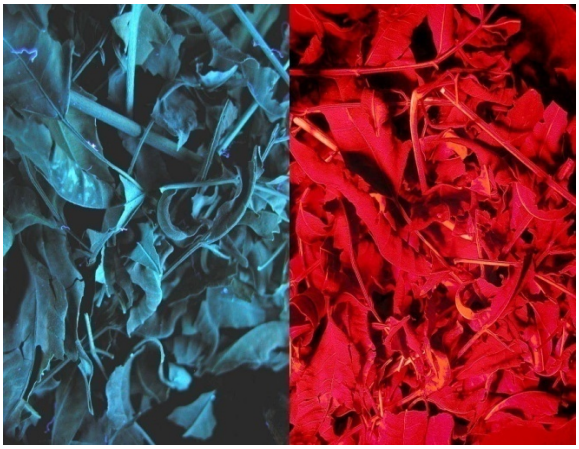

Fig. C2. Dried aerial parts under UV & IR.

Table 1. Comparative analysis of *Azadirachta indica* and *Melia azedarach* by using taxonomic and medicinal features.

No.	Characters	*Azadirachta indica* A.Juss.	*Melia azedarach* L.
01.	Nomenclature	English Names: Indian Lilac, Neem, Margosa Tree Local Names: Nim, Neem, Nimba, Azad Dirakht Trade Names: Neem, Nim, Nim Mehndi, Barg-e-Neem	English Names: Bead Tree, Chinaberry, Persian Lilac Local Names: Darek, Bakain, Mallan Nimb Trade Names: Persian Lilac, Neem
02.	Geographic distribution	In Pakistan: Sargodha, Sindh, Lower Balouchistan, Pind Dadan Khan and Jhelum. In World: Tropical and subtropical regions, India, Pakistan, Bangladesh, Burma, Sri Lanka, Nepal, Malaysia, Indonesia, Middle East, Africa and Nigeria	In Pakistan: Attock, Hazro, Mianwali, Abbotabad, Hazara, Peshawar, Mardan, Rawalpindi, Islamabad, Chakwal, Jhelum and Sind. In World: Indo-China, Southeast Asia, Australia, India, Pakistan, Nigeria, Bangladesh, Indonesia, Malaysia, Iran, Iraq, Brazil, China, Africa, Saudi Arabia, USA and Afghanistan
03.	Occurrence & habitat	Common in arid and semi-arid, tropical and subtropical parts of the world. It grows best on neutral and alkaline soils.	Common tree of subtropical climatic zone. Deep fertile, sandy loam soils support the growth of this tree. Commonly occurs in arid areas of Pakistan
04.	Morphology	Fast growing, 10-20 m tall, evergreen but deciduous in drought conditions. Widespread crown, up to 12-18 m diameter, dense branches, branch length up to 10 m, bark color dark brown. Leaves opposite, pinnate, 20-42 cm long, leaflets 2-7 cm long, light green, exstipulate, short petiole, leaflets lanceolate, acuminate apex, serrate margin. Inflorescences axillary, drooping panicle, flowers 4-7 mm long and 6-10 mm wide, subtended by minute bracts, flowers bisexual, actinomorphic, pentamerous, whitish, scented, calyx and corolla imbricate. Fruit a drupe, glabrous, 1-2.9 cm, oval, white, seed testa glabrous, brown (Fig. A1).	Deciduous tree, 10-45 m tall, spreading and rounded crown, bark greenish brown and grey. Leaves alternate, 25-50 cm long, bipinnate or sometime tripinnate, petiole long, leaflets light green, 3-11 in number, serrate margins, with a pungent odor. Inflorescences axillary, panicle up to 25 cm long, flowers 3-6 mm, fragrant, whitish petals, pentamerous, each petal 5-lobed, 0.9 cm long, pubescent; staminal tube purple to blue-brown, 0.6 cm long. Fruit a yellow drupe, round, about 15 mm in diameter, smooth, slightly fleshy. Seed oblongoid, 4.5 mm x 15 mm, smooth, brown (Fig. A2).
05.	Palynology	Pollen monad, shape in polar view circular, polar diameter 34.37 µm (33.75-35 µm), polar length 36.25 µm (35-37.5 µm), pollen shape in equatorial view spheroidal, equatorial diameter 30 µm (27.5-32.5 µm), and equatorial length 36.25 µm (30-42.5 µm), P/E ratio 1.14, exine thickness 2.5 µm (1.25-3.75 µm).	Pollen monad, shape in polar view semi-circular, polar diameter 31.52 µm (29.55-32.44 µm), polar length 34.36 µm (36-39.5 µm), pollen shape in equatorial view rhomboidal to spheroidal, equatorial diameter 34 µm (28.6-39.5 µm), and equatorial length 39.35 µm (29.5-45.5 µm), P/E ratio 1.13, exine thickness 2.4 µm (1.38-3.22 µm).
06.	Leaf epidermal anatomy	**Adaxial surface:** Ordinary epidermal cells pentagonal, hexagonal and variously shaped. Length 25µm (20-30µm), breadth 15µm (10-20µm). Anomocytic type of stomata, length 16.25µm (15-17.5µm), width 17.5µm (15-20µm), length of guard cells 26.25µm (22.5-30µm), breadth 22.5µm (20-25µm). Stomatal complex 26µm (24.5-28µm) long, 47.5µm (45-50.5µm) wide. Subsidiary cells 20.5µm (15-25.5µm) long, 12.5µm (10-15µm) wide. Trichomes long and pointed at end. Length of trichomes 150µm (87.5-212.5µm), width of trichomes 18.75µm (12.5-25µm), base of trichomes globose or rounded in shape, length of base 26.25µm (20-32.5µm), breadth 13.12µm (12.5-13.75µm). **Abaxial surface:** Ordinary epidermal cells variously shaped. Length of ordinary epidermal cells 20µm (15-25µm), breadth 12.5µm (10-15µm). Stomata irregularly oriented. Anomocytic type of stomata, length 20µm (17.5-22.5µm), breadth 17.5µm (15-20µm). Length of guard cells 25µm (22.5-27.5µm), width 17.75µm (18-20.5µm). Stomatal complex 25.75µm (23.5-28µm) long and 46.5µm (44.5-48.5µm) wide. Subsidiary cells 18µm (14-22.5µm) long and 10.5µm (8-12µm) wide. Trichomes absent (Fig. F1, G1, H1 & J1).	**Adaxial surface:** Ordinary epidermal cells variously shaped. Length of ordinary epidermal cells 32.5µm (30-35µm), breadth 19.16µm (12.5-30µm). Stomata irregularly oriented and very abundant. Anomocytic type of stomata, length 25µm (22.5-27.5µm), stomata 18.75µm (17.5-20µm), length of guard cells 22.5µm (20-25µm), breadth 8.33µm (5-10µm). Stomatal complex 19.5µm (17.5-22.5µm) long, and 25.75µm (24.5-27.5µm) wide. Subsidiary cells 21.5µm (20-30.5µm) long, 15.5µm (13-18.5µm) wide. Trichomes absent. **Abaxial surface:** Ordinary epidermal cells variously shaped. Length of ordinary epidermal cells 29µm (25.5-32.5µm), breadth 14.25µm (12.5-16µm). Stomata irregularly oriented and very abundant. Anomocytic type of stomata. Length of stomata 21µm (19.5-22.5µm), width 16.5µm (15-18µm). Length of guard cells 20.25µm (18-22.5µm), breadth 5.5µm (3-8µm). Stomatal complex 21.5µm (18-23.5µm) long, 27µm (25-28.5µm) wide. Subsidiary cells 23µm (18-28.5µm) long, and 14.5µm (12-17.5µm) wide. Trichomes absent (Fig. F2, G2, H2 & J2).

Table 1. (Contd.).

No.	Characters	*Azadirachta indica* A.Juss.	*Melia azedarach* L.
07.	Traded part & status	Leaves commonly traded throughout the world. In Pakistan dried leaves are 60-90 rupees (USD $0.5-1) per kg.	Generally not traded in the Indo-Pak Subcontinent. In markets throughout Pakistan neem leaves are adulterated with the leaves of *Melia azedarach*.
08.	Organoleptgraphy	Herbal dried raw material includes a mixture of leaves, branches and flowers. Branches are light brown in color and have ridges and furrows. Branches are hard, cylindrical and have a rough surface. There are reddish points (lenticels) visible on branches. The inside of branches is light yellowish in color. Branch length ranges from 6-12 cm. Breadth ranges from 0.3-1.8cm. Leaves are green in color with brownish prominent veins. Leaves cuspidate. Leaf length ranges from 3.6-5.3 cm. Leaves have a bitter taste and an herbal odor. Flowers are small in size, from 0.5-2 cm, and creamy in color (Fig. B1).	Dried aerial parts are bitter in taste and odourless. Branches are cylindrical, hairy and light green in color. They have ridges and furrows. Size of branches ranges from 14-19 cm. Leaves are green in color and pointed. They have dentate margins. The size of leaves ranges from 2.5-3.5 cm in length. The flowers are actinomorphic with a staminal tube. Externally flowers are light pink to purplish in color and the lower part is white in color. Size of flowers is 2-6 cm. Fresh fruit are globose while dried ones become elliptical (Fig B2).
09.	Part use	Aerial parts, twigs, bark, seeds and leaves	Leaves & seeds
10.	Medicinal uses	Diabetes, skin diseases, sores, malaria, rheumatism, digestive complaints, hepatitis, ulcer, blood purifier, leprosy, eye problems, piles, cough, asthma.	Diabetes, blood purifier, skin diseases and leprosy.
11.	Indigenous herbal recipes	According to a young lady in Village Pethi (Punjab), the powdered fruit is equally mixed (10 g) with rocket seed oil (10 ml) and applied 2-3 weeks daily at hair roots. This recipe is recommended for hair cleaning and to kill lice. Dried leaf powder (3 g) is mixed with 2-5 drops of rose extract and this paste is useful for removing freckles on the face. According to the elder people and herbalist in district Attock (Punjab), leaves of the plant are dried under shade, 5-7 dried leaves are soaked in water in a new earthen pot for a night and filtered in early morning. This is drunk for a month daily to reduce alanine aminotransferase (ALT) and cure hepatitis B without any side effects. According to a local woman in the salt range region (Punjab), dried and ground leaves of *Azedirachta indica* are mixed with three teaspoons of *Curcuma longa* powder. To prepare a paste a few drops of lemon juice are added. The paste is used as a facial mask to enhance the beauty of young girls and married ladies. The leaves of the plant are boiled in water and the extract is used to wash wounds. It acts as antiseptic. The leaves are crushed and then applied on wounds. Branches are used as a toothbrush according to an old man. In province Sindh, fresh juice from leaves is commonly used for reducing blood glucose levels and for blood purification. Local communities of this area call Indian lilac their blessing tree for poor people to cure all diseases at their homes.	1-2 teaspoons of fresh leaf extract is taken daily before breakfast for diabetes and blood purification. 5-8 g fresh leaves are crushed and mixed with one glass of water. This extract is recommended for 15-20 days daily in the summer to cure pimples and boils, purify the blood and to reduce blood glucose levels. Fresh fruits are dried in shade, and then the pericarp and mesocarp are removed and ground into powder. ½ tablespoon of powder with water is taken for a month to reduced blood glucose levels among the local communities of remote villages in Punjab province.
12.	Toxicity	Bitter in taste. Recommended doses are safe and without side effects. Excess amount may cause certain problems.	Bitter in taste but no side effects. Excessive use may cause dryness of the body. The taste of the leaves not as bitter as *Azadirachta indica* (Neem).

Table 2. Fluorescence analysis and solubility tests (Cold method) of leaf powdered drug of *Azadirachta indica* in various solvents.

No.	Treatments	Under visible light	Under short wavelength (UV) 254 nm	Under long wavelength (UV) 365 nm	On filter paper (under short wavelength UV)	On filter paper (under long wavelength UV)	Solubility Analysis
1.	Dried Plant Powdered	Green	Leaf green	Leaf green	-	-	-
2.	Powdered drug+50% KOH	Dark green	Brownish black	Blackish green	Yellow	Yellowish brown	Soluble
3.	Powdered drug+10% aq. $FeCl_3$	Greenish black	Black	Greenish black	Golden brown	Dark brown	Slightly soluble
4.	Powdered drug+dH_2O	Leaf green	Black	Blackish green	Pink	Pinkish brown	Slightly soluble
5.	Powdered drug+HCl Conc.	Dark green	Black green	Black green	Yellow brown	Golden brown	Soluble
6.	Powdered drug+HCl 50%	Leaf green	Greenish brown	Brown	Pinkish brown	Pink	Slightly soluble
7.	Powdered drug+H_2SO_4 Conc.	Black	Black	Black	Brown	Reddish brown	Insoluble
8.	Powdered drug+H_2SO_4 50%	Brownish black	Black	Dark brown	Brown	Pinkish brown	Insoluble
9.	Powdered drug+HNO_3 Conc.	Reddish brown	Dark brown	Blackish brown	Brown	Brown	Soluble
10.	Powdered drug+HNO_3 50%	Red	Brown	Chocolate brown	Pinkish brown	Reddish brown	Slightly soluble
11.	Powdered drug+Conc. CH_3OH	Leaf green	Greenish black	Reddish brown	Yellow	Yellow	Soluble
12.	Powdered drug+CH_3OH50%	Leaf green	Leaf green	Light green	Pinkish brown	Pink	Slightly soluble
13.	Powdered drug+Conc. $CHCl_3$	Leaf green	Leaf green	Reddish green	Brownish green	Pinkish green	Soluble
14.	Powdered drug+$CHCl_3$ 50%	Leaf green	Signal green	Reddish green	Pinkish brown	Pink	Soluble
15.	Powdered drug+Conc. C_2H_5OH	Grapes green	Leaf green	Reddish green	Pink	Pink	Soluble
16.	Powdered drug+C_2H_5OH 50%	Dark green	Black	Blackish green	Pink	Pinkish brown	Soluble
17.	Powdered drug+Conc. CH_3COOH	Leaf green	Leaf green	Reddish brown	Light pink	Pink	Slightly soluble
18.	Powdered drug+CH_3COOH 50%	Leaf green	Dark green	Brownish green	Pink	Pinkish brown	Slightly soluble
19.	Powdered drug+Conc. C_6H_6	Golden	Leaf green	Red	Pink	Pink	Soluble
20.	Powdered drug+C_6H_6 50%	Light green	Golden	Leaf green	Pink	Pinkish yellow	Slightly soluble

Table 3. Fluorescence analysis and solubility tests (Hot method) of leaf powdered drug of *Azadirachta indica* in various solvents.

No.	Treatments	Under visible light	Under short wavelength (UV)	Under long wavelength (UV)	Solubility Analysis
1.	Powdered drug+50% KOH	Dark green	Black	Reddish black	Soluble
2.	Powdered drug+10% aq. $FeCl_3$	Dark green	Black	Greenish black	Soluble
3.	Powdered drug+dH_2O	Light green	Blackish green	Dark green	Slightly soluble
4.	Powdered drug+HCl Conc.	Blackish green	Black	Black	Soluble
5.	Powdered drug+HCl 50%	Dark green	Greenish black	Greenish black	Slightly soluble
6.	Powdered drug+H_2SO_4 Conc.	Brownish black	Black	Brownish black	Insoluble
7.	Powdered drug+H_2SO_4 50%	Black	Dark green	Dark brown	Insoluble
8.	Powdered drug+HNO_3 Conc.	Orange brown	Brown	Blackish brown	Soluble
9.	Powdered drug+HNO_3 50%	Orange	Orange brown	Brown	Soluble
10.	Powdered drug+Conc. CH_3OH	Green	Greenish brown	Reddish brown	Soluble
11.	Powdered drug+CH_3OH50%	Golden	Dark green	Leaf green	Slightly soluble
12.	Powdered drug+Conc. $CHCl_3$	Leaf green	Green	Reddish green	Soluble
13.	Powdered drug+$CHCl_3$ 50%	Leaf green	Dark green	Reddish green	Soluble
14.	Powdered drug+Conc. C_2H_5OH	Spring green	Green	Red	Soluble
15.	Powdered drug+C_2H_5OH 50%	Leaf green	Blackish green	Reddish green	Soluble
16.	Powdered drug+Conc. CH_3COOH	Leaf green	Greenish brown	Reddish brown	Slightly soluble
17.	Powdered drug+CH_3COOH 50%	Brown	Greenish black	Brownish black	Slightly soluble
18.	Powdered drug+Conc. C_6H_6	Leaf green	Black brown	Reddish brown	Soluble
19.	Powdered drug+C_6H_6 50%	Golden	Leaf green	Dark green	Soluble

Table 4. Fluorescence analysis and solubility tests (Cold method) of leaf powdered drug of *Melia azedarach* in various solvents.

S. No.	Treatments	Under visible light	Under short wavelength (UV) 254 nm	Under long wave length (UV) 265 nm	On filter paper (under short wavelength UV)	On filter paper (under long wavelength UV)	Solubility analysis
1.	Dried leaf powdered	Leaf green	Golden	Leaf green	-	-	-
2.	Powdered drug +50% KOH	Golden	Leaf green	Dark green	Yellowish brown	Golden yellow	Slightly soluble
3.	Powdered drug+10% aq. $FeCl_3$	Golden brown	Black	Blackish green	Yellow	Dark yellow	Insoluble
4.	Powdered drug+dH_2O	Dark green	Black	Charcoal	Pinkish yellow	Pinkish brown	Slightly soluble
5.	Powdered drug+HCl Conc.	Dark green	Dark green	Greenish black	Yellowish green	Yellow	Slightly soluble
6.	Powdered drug+HCl 50%	Off white	Grayish green	Green	Yellow	Pink	Insoluble
7.	Powdered drug+H_2SO_4 Conc.	Brownish black	Black	Black	Brownish green	Black	Colloidal
8.	Powdered drug+H_2SO_4 50%	Black	Black	Black	Brownish green	Dark green	Soluble
9.	Powdered drug+HNO_3 Conc.	Golden brown	Dark brown	Blackish brown	Greenish brown	Yellowish brown	Soluble
10.	Powdered drug+HNO_3 50%	Off white	Mustard	Grayish	Pinkish brown	Pink	Soluble
11.	Powdered drug+Conc. CH_3OH	Green	Leaf green	Green	Pink	Pink	Slightly soluble
12.	Powdered drug+CH_3OH50%	Golden	Green	Leaf green	Pink	Pinkish brown	Slightly soluble
13.	Powdered drug+Conc. $CHCl_3$	Light green	Golden	Yellowish pink	Color less	Colorless	Slightly soluble
14.	Powdered drug+$CHCl_3$ 50%	Leaf green	Leaf green	Light green	Light pink	Pink	Soluble
15.	Powdered drug+Conc. C_2H_5OH	Green	Green	Reddish green	Light pink	Colorless	Soluble
16.	Powdered drug+C_2H_5OH 50%	Leaf green	Green	Lake green	Pinkish brown	Pinkish brown	Slightly soluble
17.	Powdered drug+Conc. CH_3COOH	Light mustard	Greenish black	Reddish brown	Yellow	Pink	Soluble
18.	Powdered drug+CH_3COOH 50%	Mustard	Blackish brown	Black	Pinkish yellow	Pinkish brown	Slightly soluble
19.	Powdered drug+Conc. C_6H_6	Pistachio green	Golden	Off white	Colorless	Light pink	Soluble
20.	Powdered drug+C_6H_6 50%	Leaf green	Gold dust	Grayish	Pink	Pink	Insoluble

Table 5. Fluorescence analysis and solubility tests (Hot method) of leaf powdered drug of *Melia azedarach* in various solvents.

No.	Treatments	Under visible light	Under short wavelength (UV) 254 nm	Under long wavelength (UV) 365 nm	Solubility analysis
1.	Powdered drug +50% KOH	Golden green	Brownish black	Greenish black	Slightly soluble
2.	Powdered drug +10% aq. $FeCl_3$	Brown	Black	Brownish black	Soluble
3.	Powdered drug+dH_2O	Leaf green	Leaf green	Dark green	Slightly soluble
4.	Powdered drug+HCl Conc.	Dark green	Black	Black	Slightly soluble
5.	Powdered drug+HCl 50%	Off white	Brownish black	Dark green	Slightly soluble
6.	Powdered drug+H_2SO_4 Conc.	Black	Black	Black	Slightly soluble
7.	Powdered drug+H_2SO_4 50%	Black	Black	Black	Soluble
8.	Powdered drug+HNO_3 Conc.	Pale cream	Beige	Mustard	Insoluble
9.	Powdered drug+HNO_3 50%	Lemon	Lemon	Pale cream	Soluble
10.	Powdered drug+Conc. CH_3OH	Leaf green	Leaf green	Green	Slightly soluble
11.	Powdered drug+CH_3OH50%	Golden	Leaf green	Light green	Insoluble
12.	Powdered drug+Conc. $CHCl_3$	Spring green	Green	Reddish green	Highly soluble
13.	Powdered drug+$CHCl_3$ 50%	Spring green	Leaf green	Leaf green	Soluble
14.	Powdered drug+Conc. C_2H_5OH	Spring green	Green	Reddish green	Slightly soluble
15.	Powdered drug+C_2H_5OH 50%	Thick green	Green	Grayish green	Soluble
16.	Powdered drug+Conc. CH_3COOH	Mustard	Black	Reddish black	Soluble
17.	Powdered drug+CH_3COOH 50%	Golden brown	Brownish black	Black	Soluble
18.	Powdered drug+Conc. C_6H_6	Spring green	Golden	Reddish green	Soluble
19.	Powdered drug+C_6H_6 50%	Leaf green	Leaf green	Light green	Insoluble

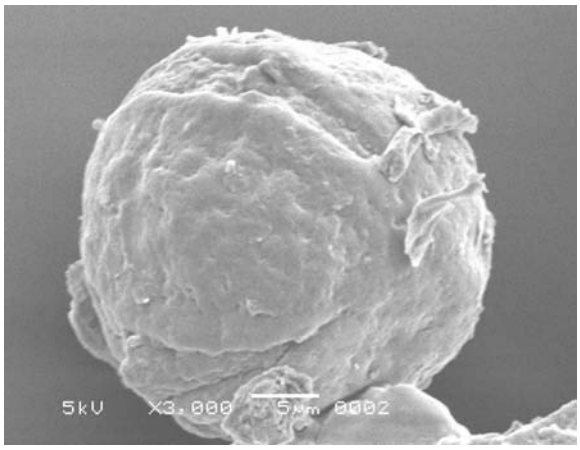

Fig. D1. Oblique Polar View of Pollen (SEM).

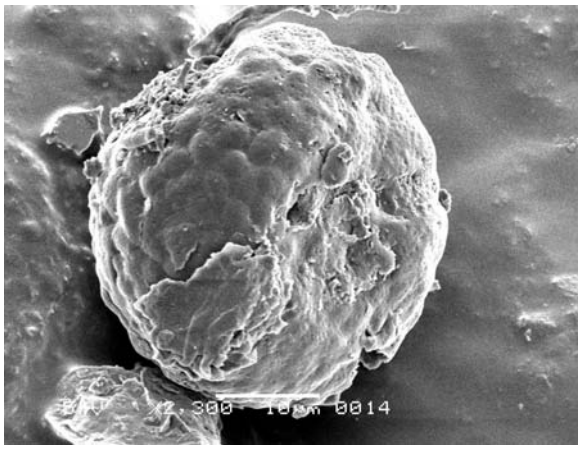

Fig. D2. Oblique Polar View of Pollen (SEM).

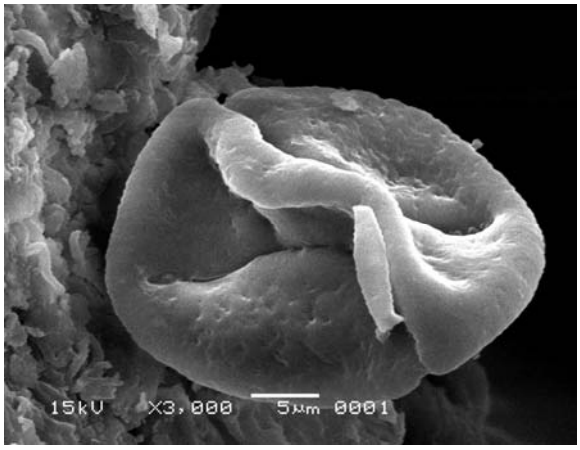

Fig. E1. Equatorial View of Pollen (SEM).

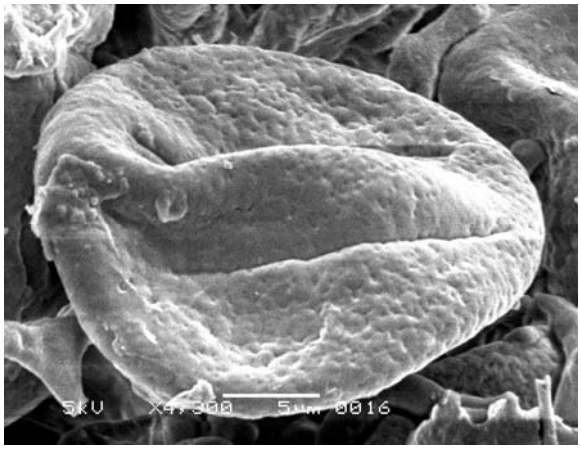

Fig. E2. Equatorial View of Pollen (SEM).

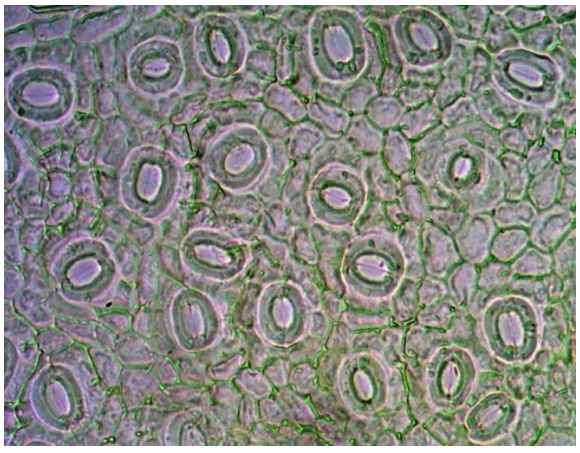

Fig. F1. Stomata & Epidermal Cells Abaxial Surface (LM-40x).

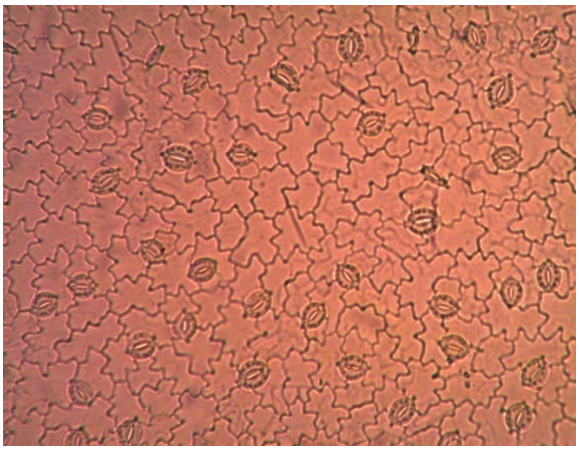

Fig. F2. Stomata & Epidermal Cells Abaxial Surface (LM-40x).

Similarly, at a microscopic level, palynological and anatomical features are often so diagnostic that they are now commonly used in routine identification of traded herbal materials. Foliar epidermal characters are taxonomically important features that include stomata, trichomes, subsidiary cells and epidermal cells; subsidiary cells, if they are distinct from the normal epidermal cells, are often the most valuable. *Azadirachta indica* has long pointed trichomes with a rounded base (Fig. G1) on adaxial surfaces. No trichomes were observed on *Melia azedarach* leaves. Similarly, numerous stomata were observed under light microscope on *Melia azedarach* leaves (Fig. F2), while they were lesser in number on *Azadirachta indica* leaves (Fig. F1).

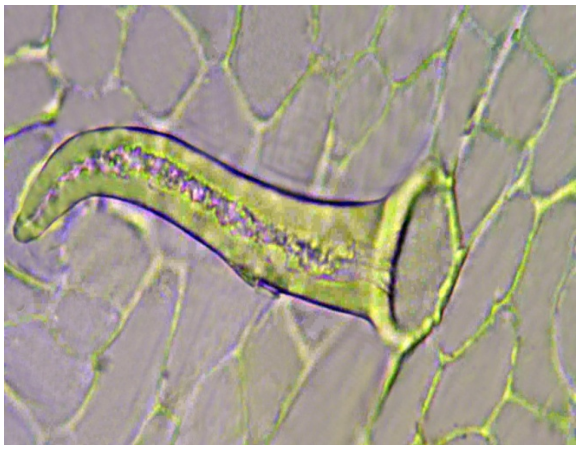

Fig. G1. Leaf surface with trichome Adaxial Surface (LM-40x).

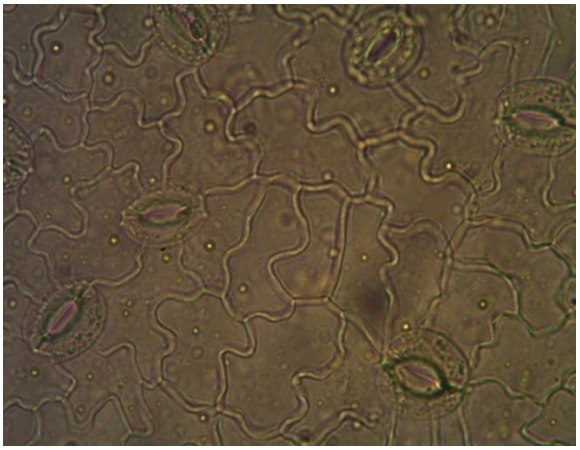

Fig. G2. Leaf surface without trichome Adaxial Surface (LM-40x).

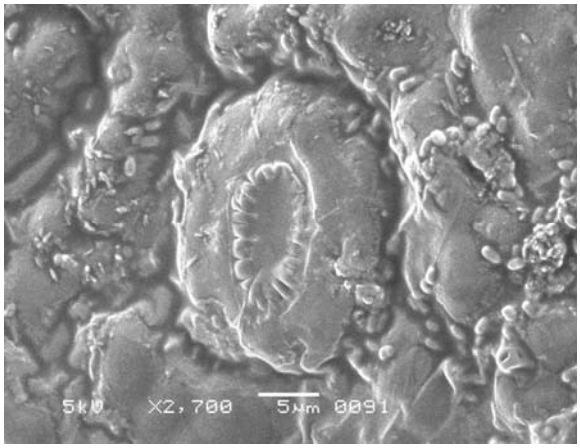

Fig. H1. Stomata Abaxial (SEM).

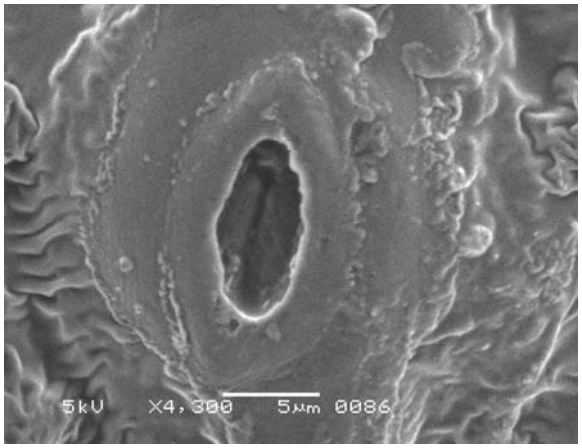

Fig. H2. Stomata Abaxial (SEM).

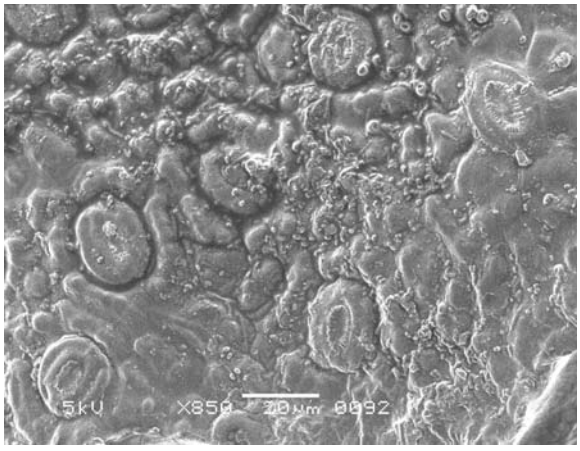

Fig. J1. Stomata (SEM).

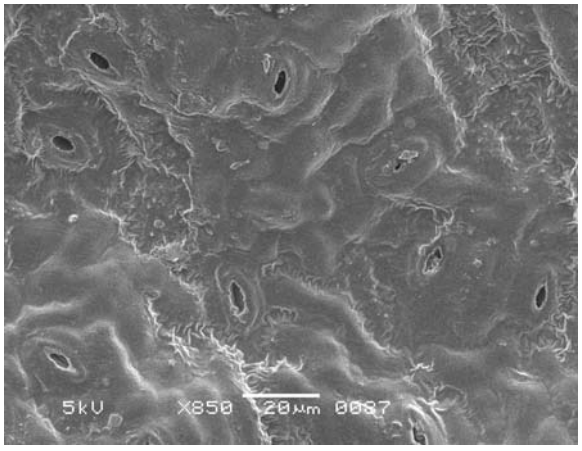

Fig. J2. Stomata (SEM).

Palynological features are very useful for authentication at a microscopic level to distinguish closely related species (Zafar *et al*., 2007). *Azadirachta indica* has a perforate sculpturing of the pollen exine with a few unevenly distributed holes or depressions, while rest of the surface of pollen is psilate (Fig. D1 & E1). *Melia azedarach* has pollen sculpturing that is perforate with numerous minute holes which are distributed over the surface (Fig. D2 & E2). On the basis of sculpturing characteristics *Azadirachta indica* can be easily distinguished from *Melia azedarach.*

It is concluded from this study that *Azadirachta indica* and *Melia azedarach* can be distinguished on the basis of morpho-palynological and foliar epidermal anatomy. Based on pharmacognostic tests, the powdered drug can be distinguished on the basis of solubility and fluorescence

analysis. Solvents like 50% KOH, 10% aq. $FeCl_3$, 50% HCl, HNO_3, CH_3COOH and C_6H_6 can be used to differentiate the powdered drug of *Azadirachta indica* and *Melia azedarach* easily (Tables 2-5). Among these distinguishing solvents, HNO_3 and C_6H_6 are recommended as a good test for authenticating the herbal drug Neem. This type of study may lead to the authentication of herbal drugs procured from markets for the correct identification of the medicinal plant ingredients.

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