Microscopic studies and preliminary pharmacognostical evaluation of *

Euphorbia neriifolia* L. leaves

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Euphorbia neriifolia Linn. (Euphorbiaceae) is commonly known as Indian Spurge Tree in English, and Sehundah in Ayurveda. Its leaves are traditionally used for treatment of various diseases like inflammation, fever, asthma, cough, wounds, ulcers, cancers and diabetes. In the present study an attempt has been made to highlight this folk herbal medicine which will assist in the identification of fresh as well as dried crude samples of leaves anatomically and pharmacognostically. The present study also deals with macroscopic, microscopic, fluorescence, phytochemical characteristics and other WHO recommended methods for standardization of leaf powder. Phytochemical screening and chromatographic studies help in determining the antioxidant potential and predominant classes of active ingredients contribute to the activity. These studies will provide referential information for correct identification and help in checking adulteration in market samples used in the preparation of various herbal medicines. These observations will also be helpful in differentiating the leaves of this species from closely related species of same genus and family.

Keywords: Euphorbia neriifolia, Microscopic, Pharmacognostic, Phytochemical Screening, Standardization, Chromatography.

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Introduction

An authentication and quality assessment of herbal material deals with the pharmacognosy that is based on macroscopic and microscopic characters. A big quantum of research works in the area of authentication of the correct plant source has been undertaken to provide means of differentiation among many available plant sources. *Euphorbia neriifolia* L. (Euphorbiaceae) is a widely distributed large succulent shrub or small tree up to 7.5 m in height, with stipular thorns and found throughout the Deccan Peninsula of India. It is commonly known as Sehundah (Ayurveda), *Ilachevikalli* (Siddha), Indian Spurge Tree, Oleander Spurge and Hedge Euphorbia. Traditionally it is used as antibacterial, antifungal, antiviral, antiparasitic, antiarthritic, antidiabetic, anticonvulsant, antioxidant, analgesic, wound healing and immunomodulatory, radioprotective, spasmodic, aphrodisiac, anticancer, purgative and limit diseases caused by Tiba (indigestion and badkhara and Tipa) and diuretic properties due to the presence of phytoconstituents. The efficacy of this plant in the treatment of various diseases necessitated the present work in order to identify the classes of natural products present in the leaf of the *E. neriifolia*. For standardization and quality assurance purpose, the following three attributes must be verified: authenticity, purity and assay. Literature survey did not provide sufficient information about pharmacognostical studies of this plant. The current work aims to contribute in solving the problems of controversial drugs prevalent in Ayurveda besides helping in laying down pharmacopoeial standards. Therefore, keeping above view in mind various macroscopic, microscopic and pharmacognostical studies on fresh and dried leaves of *E. neriifolia* were carried out in present study.

Materials and Methods

Procurement and authentication

*Euphorbia neriifolia* leaves were collected from Medicinal garden of Banasthali University, Banasthali, Rajasthan, India, in the month of September-November 2009 and authenticated by Botanist of Krishi Vigyan Kendra, Banasthali University, Banasthali.

Preparation of leaves extracts

Shade dried powder was extracted by macerating 50 g in 250 mL of ethanol (70% v/v) for one week with occasional stirring. Coarse powder (50 g) was also defatted in 250 mL of ethanol (70% v/v) by using Soxhlet.
apparatus. Successive extraction with different solvents in their ascending order of polarity was also carried out using Soxhlet apparatus. The macerated and Soxhleted mixture was filtered, evaporated and stored at 4°C in air tight container and was used for further studies.\textsuperscript{12}

Macroscopic and microscopic studies

Characters were examined according to Brain and Turner.\textsuperscript{13} Quantitative microscopy include surface constants like stomatal number, stomatal index, veins, vein-islets and vein terminations were studied by using camera lucida. Various diagnostic characters of fresh leaves and leaf powder were studied by microscopic analyses with or without staining.\textsuperscript{14-17}

Pharmacognostical analysis

Pharmacognostical values such as the foreign organic matter, percentage of total ash value, acid insoluble & water soluble ash value, moisture content and extractive values were performed according to the WHO guidelines on quality control methods for medicinal plant materials.\textsuperscript{18-21} Chemical test and fluorescence analysis were also studied.\textsuperscript{15, 19, 22, 23}

Preliminary phytochemical screening

Preliminary qualitative phytochemical screening of all the extracts for the detection of various active ingredients was carried out by using standard conventional procedures.\textsuperscript{13, 23, 24}

Chromatographic fingerprinting

Thin Layer Chromatography (TLC) of all the extracts was carried out in various solvents at 30°C using Silica gel G as adsorbent and the \( R_f \) values were determined. The selected mobile phase was used for the high performance thin layer chromatography (HPTLC) densitometry analysis of different extracts of EN were also performed.\textsuperscript{25}

Results

Organoleptic and macroscopic evaluation

Leaves of \textit{E. neriifolia} are succulent, deciduous, terminal on branches, fleshy, stipular thorns in pairs, 3-5 mm long. Macroscopically, the fresh young leaves are simple, dark green in colour having leathery texture, cuneate shaped, apex sub-acute and base acute, margins entire (even, smooth throughout) without toothning, surface glabrous, venation reticulate and average leaf size 8-24 ± 2 cm (length) and 4-8 ± 2 cm (breadth) & 1.3±0.2 mm (thickness) with pointed and acute tip. The length of reduced petiole 1.5-2.5 cm (Plate 1).

Powder microscopy

The fine powder was mounted in glycerin and stained with iodine, phlorogucinol + conc. HCL and Sudan III. Observed features revealed that the leaf powder contains numerous idioblastic, rosette, square, prismatic and acicular shaped calcium oxalate crystals and starch grains, both simple and compound. The powder also showed the presence of well arranged annular vessels, anomocytic stomata, unicerrate multicellular trichome with blunt tip, epidermal cells, spongy parenchyma, xylem parenchyma, vittae- volatile contain schzogenous cells, polyhedral or sharp angles typed starch grains and lignified xylem fibers (Plates 2-6). After treatment with HCL calcium oxalate crystals changed into needle shaped crystals (Plate 2).

Plate 1—(a-c)- Organoleptic and morphological features of \textit{E. neriifolia} leaves

Plate 2—(a-e)- Different types of calcium oxalate crystals present in powder of \textit{E. neriifolia} leaves
Leaf microscopy

Transverse section of leaf showed the single layered thick rectangular or tubular adaxial epidermal cells. Single to double layered abaxial epidermis with circular to rectangular epidermal cells (Plate 7 a-c). Mesophyll tissue was differentiated into two to three layered adaxial zones of radially elongated palisade cells and wider abaxial spongy mesophyll cells revealed the differentiated dorsiventral lamina (Plate 7d). The spongy mesophyll had wide air-chambers and partition filaments formed by lobed and interconnected 6-9 layered spongy parenchyma cells. Midrib region slightly raised on the abaxial side whereas broadly semi-circular on the adaxial side. Midrib composed of epidermis, collenchyma and spongy parenchyma cells (Plate 7b). In certain regions of epidermis, five celled unicereate trichomes with blunted tip (Plate 7a) and glandular trichomes with bicellular head (Plate 7 c) were embedded.

Vascular bundles (VB) prominent towards the ventral side and covered with endodermis. The vascular bundles consist lignified xylem (pink colour) whereas non-lignified phloem (Plate 8a). Parenchymatous cells of leaf

Plate 3 & 4— (a-e)- Different types of trichomes, Anisocytic stomata present in powder of *E. nerifolia* leaves

Plate 5 & 6— (a-d)- Annular xylem trachieds, lignified fibre & vessels, - Xylem parenchyma cells, spongy parenchymatous cells seen in the powder of *E. nerifolia* leaves

Plate 7 & 8— (a-d)- Transverse sections of *E. nerifolia* leaf, a) UE with unicellular trichomes, b) LE with Col & mesophyll SP, c) UE with glandular trichomes (GT), d) Palisade parenchyma (PalP); 8a a.Vascular bundle (VB), b. Transfusion tissue
showed the presence of transfusion tissue after stained with acid (Plate 8b). Between loosely arranged spongy cells rosette, octahedral and thick calcium oxalate crystals were seen (Plate 9a). These crystals bearing cells were distinct from the neighboring mesophyll cells were called idioblasts. Direct stained with potassium iodide the spongy parenchyma showed the presence of starch granules (Plate 9b). The spongy mesophyll had wide air-chambers and partition filaments formed by lobed and interconnected 6-9 layered spongy parenchyma cells (Plate 9c).

*E. neriifolia* leaf surface showed the anomocytic type of stomata that were covered with guard cells surrounded by 2-3 subsidiary cells followed by polygonal epidermal layers (Plate 10b). Adaxial surface contains more stomata in comparison to abaxial surface of leaf (Plate 10a-c). Some covering trichomes with collapsed cell were also seen in upper stomata (Plate 10b). Leaf surface also showed the presence of veins, vein islets and vein terminations (Plate 10d). The primary and secondary veins branched profusely and gave rise to ultimate veinlets. The vein islets were distinct, small and squarish or rectangular. Each vein islet had one or two vein terminations and filled with areoles. Leaf constants such as stomatal number, stomatal index, veinlet terminations and vein-islet number are measured and shown in Table 1.

**Pharmacognostical analysis**

Foreign organic matter recorded in the powdered plant material was 0.87±0.03% and the percentage of extractive values of *E. neriifolia* was found to be 14.32±0.04 in alcohol (ethanol) and 26.31±0.12 in

<table>
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<th>Variables</th>
<th>Abaxial surface Value (in 1 mm² area)</th>
<th>Adaxial Surface Value (in 1 mm² area)</th>
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<tr>
<td>Stomatal number</td>
<td>116±5 mm²</td>
<td>52±5 mm²</td>
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<tr>
<td>Stomatal index</td>
<td>12.88%</td>
<td>11.60 %</td>
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<tr>
<td>Epidermal cells</td>
<td>784±10 mm²</td>
<td>396±10 mm²</td>
</tr>
<tr>
<td>Vein islet number</td>
<td>33±5 mm²</td>
<td></td>
</tr>
<tr>
<td>Veinlet termination number</td>
<td>16±5 mm²</td>
<td></td>
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Plate 9— (a-c)-TS of *E. neriifolia* leaf a) Idioblastic calcium oxalate crystals & simple starch granules, b) Polyhedral sharp angles type starch granules, c) Spongy parenchyma

Plate 10— (a-c)- Upper & lower stomata (10 X & 40X), d) Vein-islet (VL) & vein termination (VT) at 40X; SC: subsidiary cells, GC: guard cells; S: stomata; EC: epidermal cells
water. The percentage of weight loss on drying or moisture content at 105ºC of EN was found to be 3.45± 0.09, respectively whereas the pH of drug was found to be slightly acidic and the values being 6.4± 0.2 in 1% and 5.8± 0.04 in 10% aqueous solution. Physical state of dry ash appeared as fine powder and the sample ash as greyish white colour. The taste of the ash was found to be pungent and the amount of total, acid insoluble and water soluble ash of EN were found to be 7.36±0.07, 0.82±0.04, and 4.54±0.11%, respectively.

The behaviour of *E. neriifolia* leaves powder upon treatment with different chemical reagents showed creamy colour when powder was as such; light yellow brown colour with distilled water; greenish yellow with picric acid; yellow brown with glacial acetic acid; radish brown with 1N HCl and 1N H$_2$SO$_4$; yellow brown with Conc. HNO$_3$; grey with ferric chloride (5%); greenish brown with iodine solution (5%); olive green with ammonia solution; golden brown with 1N NaOH; dark brown with potassium dichromate; yellowish orange with HNO$_3$ + NH$_3$ solution; pale green with methanol and ethanol; yellow green colour with toluene. Likewise the fluorescence characteristics of powdered leaves after treatment with different reagents emitted various colour radiations under ultraviolet light (Table 2). The fluorescence characteristics of different extracts of leaf was also studied under ordinary and UV light (366 nm), wherein the leaf extracts showed the visibility of varying colours which are tabulated in the Table 3.

The preliminary phyto–profiling for the leaves extracts was carried out and the consistency was found to be sticky, dry and oily. The percentage yield (w/w) of the extracts were also analysed (Table 4), and the highest yield was found to be in aqueous extract (17.89%) in sequential soxhletion method and in hydro-ethanolic extract (20.02 %) in direct method.

### Preliminary phytochemical screening

The results of preliminary phytochemical screenings of different extracts of leaves mainly revealed the presence of proteins, alkaloids, glycosides, flavonoids, phenolics, saponins and terpenoids in appreciable, moderate and trace amount (Table 5). Our results also revealed that the plant possesses proteins and amino acids in negligible amount. This indicates that, the presence of secondary metabolites may have suppressed the activity of proteins. In addition, the solvent might have also denatured the proteins because of which it is detected in fewer amounts in all extracts.

### Chromatographic analysis

The resolution of different kinds of chemical components and the marker compound has been separated using TLC and HPTLC with the standards,
and the $R_f$ values were calculated in order to standardize the drug for its identity, purity and strength. TLC fingerprinting of different extracts of *Euphorbia neriifolia* leaf was done and phytoconstituents were separated in eight different mobile phase of varying polarity. Among all, chloroform: methanol (9.5: 0.5) mobile phase was suitable for chloroform extract, ethylacetate: methanol: water (7.5: 1.25: 0.5) for ethyl acetate extract and chloroform: ethanol: water (8:2:1) and n-butanol: acetic acid: water (4: 1: 7) were found to be most appropriate solvent system for separation of flavonoid phytoconstituents for ethanol extract. Iodine vapours were used as a developer. TLC studies of chloroform and ethyl acetate extract revealed 7 spots whereas ethanol extract revealed 8 spots at varying $R_f$ values depicted in Table 6 and Plate 11.

The n-butanol: methanol (9.5: 0.5) showed good separation of the flavonoids from petroleum ether extract of *Euphorbia neriifolia* leaves. The petroleum ether extract displayed the presence of 10 types of
flavonoids with 10 different $R_f$ values ranging from 0.18 to 0.95 (Table 7). The chromatogram was observed in UV chamber at 254 nm in absorbance and at 366 nm in fluorescence modes (Plate 12). The characteristics pattern of petroleum ether extract showed fine separated pattern of bands and served as idiosyncratic fingerprint for qualitative evaluation of leaf.

**Discussion**

Today sophisticated modern research tools for evaluation of the plant drugs are available but microscopic method is still one of the simplest and cheapest methods to establish the correct identity of the source materials.\(^{26}\) Fluorescence is an important phenomenon exhibited by various chemical constituents present in plant material. If the substances themselves are not fluorescent, they may often be converted into fluorescent derivatives by applying different reagents hence some crude drugs are often assessed qualitatively in this way and it is an important parameter of pharmacognostical evaluation.\(^{27}\).

According to the World Health Organization (WHO, 1998)\(^{21}\), the macroscopic and microscopic description of a plant is the first step to establish the identity and the degree of purity of such materials and
should be carried out before any tests are undertaken (Neeli et al., 2008). Macroscopical evaluation is a qualitative evaluation based on the study of morphological and sensory profiles of drugs and serve as diagnostic parameters. In the present study entire leaf margin and anisocytic type stomata is noticed in *E. neriifolia* leaf. The microscopical studies of the transverse sections showed presence of unicellular blunted and glandular trichomes. Numbers of leaf measurements which included stomatal number, vein islet number, veinlet termination number and stomatal Index were used to study microscopic features (Evans, 1998).

The microscopical studies of the transverse sections showed presence of unicellular blunted and glandular trichomes. Numbers of leaf measurements which included stomatal number, vein islet number, veinlet termination number and stomatal Index were used to study microscopic features (Evans, 1998). The present macroscopic and histological observations of leaves thus provide useful information for quality control parameters for the crude drug. Powder, quantitative and fluorescence standards provide valuable information to substantiate and authenticate the phytomedicine.

Estimation of ash values is also a significant parameter for the detection of nature of material, which is added to the drug for the purpose of adulteration, impurities and determination of authenticity, quality and purity of test sample. The ash values usually represent the inorganic salts present in the drug/test sample and is the residue remaining after incineration. Total ash values of leaves indicate the inorganic composition or earthy materials and other impurities present along with the plant material. The total ash value was relatively higher which may be due to high content of carbonates, phosphates, silicates and silica.

Extractive values determination were primarily useful for the identification of exhausted drugs. The amount of the extract that drug yields in a solvent is often an approximate measure of the amount of certain constituents that the drug contains. The water soluble extractive value was indicating the presence of sugar, acids and inorganic compounds and the alcohol soluble extractive values indicate the presence of polar constituents like phenols, steroids, glycosides and flavonoids.

Determination of the moisture content of the drugs used in Ayurvedic system of medicine is very important. The higher or lower percentage shows that the drug was resorted in humid, wet or dry climate. Excessive moisture may favour the growth of fungal...
or may cause other micro-organic contamination that may results in the deterioration of drug. Percentage of loss of weight on drying indicates the loss of volatile substances along with water, which is determined by subtracting the moisture content of the powder drug from the loss of weight on drying. So, the loss on drying percentage was also determined. The less value of moisture content could prevent bacterial, fungal or yeasts growth\textsuperscript{21,30}. The pH value of powder drug is also an important parameter. The drugs in the opposite pH are unionized and absorbed rapidly from stomach.

The micro-chemical tests of the drug were carried out with different concentrated mineral acids. The colours produced by these reagents represent the presence of active constituents. The fluorescence character of any powdered character of any powdered drug is very distinctive and helpful distinguishing features for the determination of a drug. The analysis of powdered drug under ultraviolet light establishes the colour of the drug, as such and after treatment with different reagents.

The presence of phytochemicals in all extracts of leaves of this species is known to be useful in the treatment of inflamed or ulcerated tissues and they have remarkable activity in cancer prevention\textsuperscript{6,8}. Flavonoids serve as health promoting compound as a results of its anion radicals\textsuperscript{31} and could prevent the accumulation of DNA damage induced by UV radiation. These observations support the usefulness of this plant in folklore remedies in the treatment of stress related ailments and as a wound healing properties\textsuperscript{35}. The plant extract was also positive for steroids which are very important compounds especially due to their relationship with compounds such as sex hormone\textsuperscript{34}. Secondary metabolites observed in this plant may be responsible for various pharmacological effects\textsuperscript{5,13}.

TLC and HPTLC methods for detection and quantification of active ingredients present in \textit{E. neriifolia} have not been reported in literature. TLC had been developed in the present work for the separation and determination of constituents of HEEN and sequential extracts of \textit{E. neriifolia} but the densitometric HPTLC had been developed for the separation and determination of flavonoids from petroleum ether extract of \textit{E. neriifolia}. Screening results clearly depicts that all extracts contain wide range of active ingredients. All mobile phases used for the chromatography were good and suitable for the extraction of flavonoids from plant extracts\textsuperscript{12}. TLC and HPTLC results of present study firmly depicted that this plants contains a wide range of flavonoids and that’s the reason behind its excellent pharmacological properties\textsuperscript{5,10,35,36}.

**Conclusion**

No detailed standardized work has been reported in literature for this plant. Leaves powder subjected for microscopic, pharmacognostical and preliminary phytochemical analysis provides relevant information which may be helpful in authentication of the crude drug and check adulteration for quality control of raw material. Chromatographic analysis showed the presence of flavonoid in extracts. The pharmacognostic parameters observed in present study, being reported for the first time adds to the existing knowledge of \textit{E. neriifolia} and be quite useful for identification, standardization, development and preparation of crude drug’s formulation and inclusion in various pharmacopoeias for treating various ailments. The current observation will also be helpful in differentiating the leaves of this species from closely related species of same genus and family.

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