Pharmacognostic and Preliminary Phytochemical Investigations on *Jatrophae curcatis semen*

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ABSTRACT

Introdution: *Jatropha curcas* L.(Euphorbiaceae) distributes in many parts of China with abundant resources. The seed (*Jatrophae curcatis semen*) of this plant is used to produce biodiesel, and the extract also has strong molluseicidal effect on *Oncomelania.* But few researches have been done about the pharmacognostic and phytochemical nature of the seed. **Methods:** The macroscopic and microscopic characters were observed by conventional methods and optical microscope. Moisture content, the alcohol soluble extractive and the fatty oil content were determined respectively. The identification and ingredient analysis were conducted by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). **Results:** The main microscopic characteristics are palisade sclereids, pillar stone cells and filiform sclereids. Moisture content should be not more than 8.0%, alcohol soluble extractive value not less than 14.0%, and content of fatty oil not less than 35.0% according to sample analysis. TLC fingerprinting of the seed extracts was developed. **Conclusion:** The results presented can provide some references for authenticity and quality control of *Jatrophae curcatis semen*.

Key word: *Jathrophae curcatis semen,* Pharmacognosy, Phytochemistry

INTRODUCTION

Jatrophae curcatis semen is the dried mature seed of *Jatropha curcas* L., which is subordinate to Euphorbiaceae. The plant is widely distributed in Yunnan, Sichuan, Guizhou, Guangdong, Guangxi province of China, and the plant is widely cultivated. The main components of the seed are fats, proteins, polypeptides, terpenes and so on[1].The fats have been extensively researched and utilized as lubricants and biodiesel[2]. The methanol extract of *J. curcatis semen* has molluscicidal effect towards vector freshwater snails, the special host of human schistosomes[3,4]. Hubei Provincial Center for Diseases Control and Prevention also reported the extract of indigenous seed possessed strong inhibition effect on *Oncomelania hupensis* by immersion method. To prevent schistosomiasis in water task, the seed has been developed into external preparations in Hubei province, and the results proved well.

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However, a scientific quality specification has not yet been established for the quality control of this crude drug. In view of rich resources and the special medicinal value, studies on the anatomical structure and preliminary physicochemical characteristics of *Jatrophae curcatis semen* were carried out, so as to provide some references of identification and quality evaluation for exploitation and utilization.

MATERIALS AND METHODS

Plant materials and reagents

The seeds of *J. curcas* L. were collected from Guangxi Autonomous Region of China and authenticated by one of the authors, Prof. Dingrong Wan,a qualified plant taxonomist.

All reagents used for testing were analytical reagents except chromatographically pure acetonitrile and purified water.

Morphological studies

Morphological studies of the crude drugs were performed at macroscopic and microscopic levels. For macroscopic ones, dried mature seeds from different geographical locations were employed. Characters of the material with

respect to shape, size, color, surface, fracture, odour and taste were researched. For microscopic ones, free-hand sections of the seeds were obtained and rendered slightly transparent. The anatomical features such as the structure of testa were observed on transverse sections under the optical microscope. Using light microscopy at appropriate magnification, the dried powder of the seeds were also observed for the diagnostic microscopical features.

Thin layer chromatographic studies

2g dried materials were sonicated with 20ml of petroleum ether (90-120°C) for 1h and filtered to remove fat-soluble impurities. Then, the residues of the samples were extracted with 20ml of ethyl acetate by sonicating for 40min and followed with filtration. The filtrate was evaporated to dryness and re-dissolved in 1ml of dehydrated alcohol pending analysis. The extracting solution of reference crude drug of *Jatrophae curcatis semen* was made by employing the method as listed above.6μl of the sample solution and reference crude drug solution, were separately loaded on a silica gel G precoated TLC plate and developed in a mixture of cyclohexane-benzene-ethyl acetate-glacial acetic acid(20:4:6:0.5) by one dimensional ascending method. After development, the plate was air-dried and sprayed with a solution of 10% sulfuric acid in ethanol, then heated at 105°C until the visualization was performed[5-7].

Quantitative determination

Moisture contents of 10 samples were determined according to the specification of Chinese Pharmacopoeia (2010 Edition, Appendix □ H). Alcohol soluble extractive value of 10 samples were obtained in 95% alcohol using cold maceration method as prescribed by Chinese Pharmacopoeia (2010 Edition, Appendix □ A).

In order to assay content of the fatty oil, 1g of coarse powders of 10 samples were separately extracted with moderate diethyl ether in soxhlet extractor for 8h.Each extract was dried in the evaporating dish with constant weight. Then, the extracts were placed in an oven at 100°C for 1h and moved into a desiccator to cool down for 30min. Finally, the extracts (fatty oil) obtained were accurately weighed and their percentages were calculated in the light of initial air-dried materials.

RESULTS

Macroscopic characteristics

The seed is elliptical or ovate with a length of 1.6-2.1cm and a width of 0.8-1.2cm. The surface is greyish-black, black or deep blackish-brown in color, and relatively rough, usually possessing many concave spots or fissures in a groove-like shape. One side presents slightly flat, mostly cracked lengthwise into a distinct deep-fissure and many fissures in a groove -like shape, whereas presents arched on the other side. One end of the seed bears a greyish-white, rhomboid hilum and a faintly protruded brownish-black caruncle or a scar of fallen caruncle. The seed has a thick, hard but fragile testa. After striping the seed coat, a layer of extremely thin, milk-white endopleura connected with endosperm, is visible. The endosperm is thickened, white and oily. The 2 cotyledons are strongly thin. The seed is odorless, possessing a slightly astringent taste and can numb the tongue durably.

Microscopic characteristics

Transverse section: The testa comprises a palisade sclereid layer, parenchyma, a pillar stone cell layer and a filiform sclereid layer. The palisade sclereid layer is a layer of palisade rectangular stone cells, arranged orderly and densely, with relatively thin lateral walls and distinctly thickened outer walls, containing yellowish-brown to blackish-brown substances. The clefts are often found in this sclereid layer, filled with parenchyma cells at the inner side. The parenchymatous tissue consists of several to 10 or more layers of parenchyma cells, with 3-4 layers of relatively small cells at the inner side, often containing clusters of calcium oxalate and laticifers. The pillar stone cell layer has a layer of small, short cylindrical or cylindrical stone cells, arranged regularly and closely, presenting yellowish-brown contents. The filiform sclereid layer is constituted by a layer of slender, fibrous stone cells, frequently curved, arranged radially and densely, containing brownish-black substances. The thin endopleura, separated from the testa, is made up of several layers of flat parenchyma cells, accompanied by vascular bundles at the inner side. The dilapidated tissue exists in the inside of endopleura, is relatively thin, and sometimes separated from endopleura. The cells of endosperm contain oil droplets and aleurone grains. The cotyledons are thin, often detached from endosperm. The parenchyma of endopleura, endosperm and cotyledons all contain a number of fine clusters of calcium oxalate. (Fig.1, 2, 3)

Powder: The powder is greyish-yellow to greyish-black in color. There are three types of sclereid. The first one is filiform sclereid, mostly in bundles, densely arranged in order, with even outer edge of outer walls. And the stone cell is rectangular, palisade-like or irregular rectangular, about 60-200μm long and 13-25μm in diameter. The walls are relatively thin on the three sides and outer wall is extremely thickened up to 20μm, in which pits and pit canals are visible, containing yellowish-brown to blackish-brown substances in lumen. The second one is the small pillar stone cell, arranged in a row, and the stone cell is

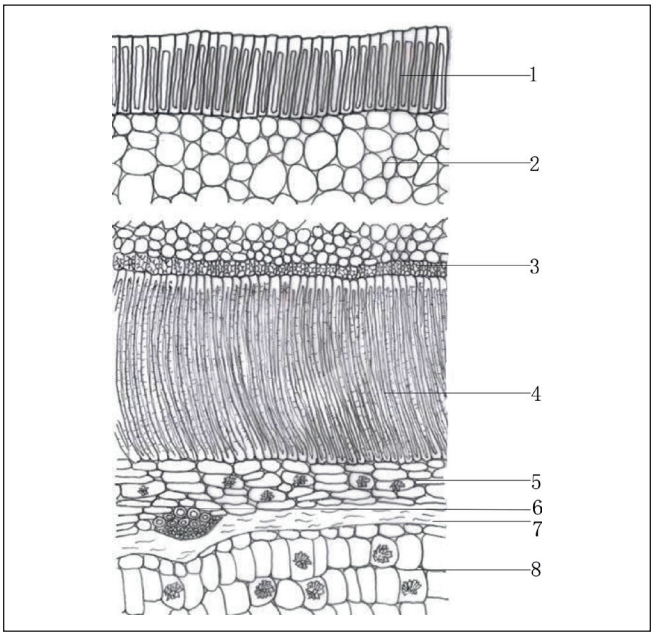

Figure 1: Detailed drawings of the transverse section of the testa from *Jatrophae curcatis semen*
1. palisade sclereid layer; 2. parenchyma; 3. pillar stone cell layer; 4. filiform sclereid layer; 5. endopleura; 6. vascular bundles; 7. diapidated tissue; 8. endosperm

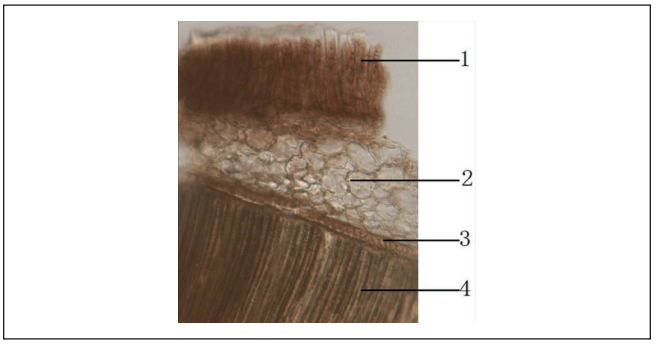

Figure 2: Microgram for the transverse section of the testa
1. palisade sclereid layer; 2. parenchyma; 3. pillar stone cell layer; 4. filiform sclereid layer

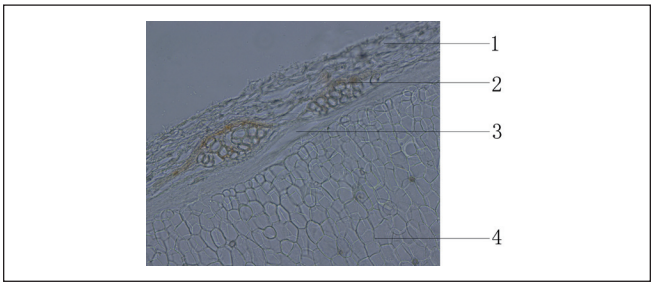

Figure 3: Microgram for the transverse section of the endopleura to endosperm
1. endopleura; 2. vascular bundle; 3. diapidated tissue; 4. endosperm

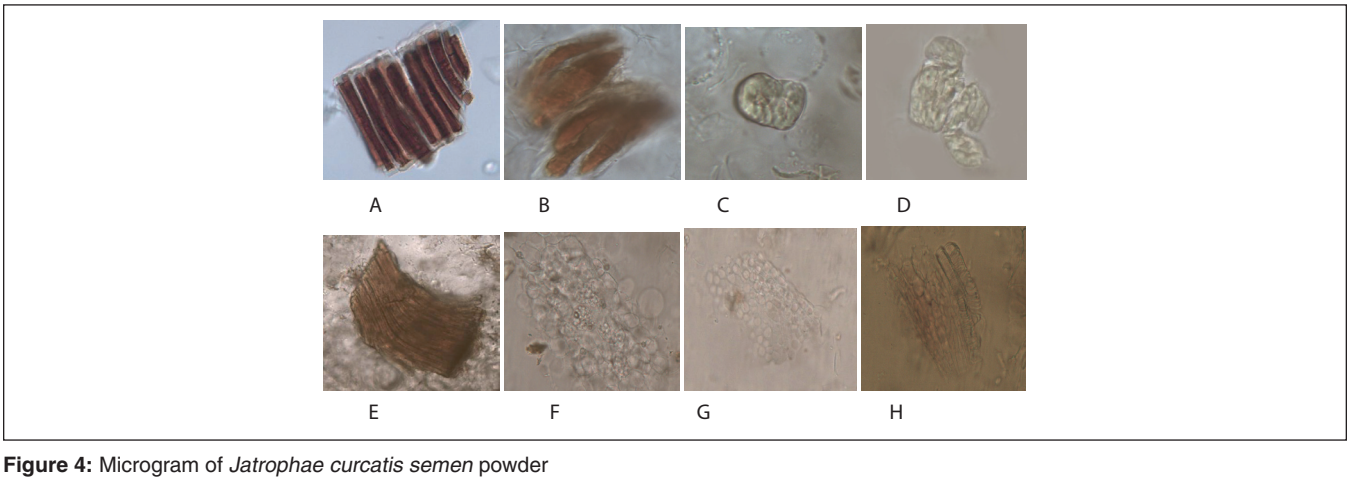

Figure 4: Microgram of *Jatrophae curcatis semen* powder
A, B. palisade sclereid; C, D. pillar stone cell; E. filiform sclereid; F. endosperm; G. cotyledon fragments; H. spiral vessel

irregular short cylindrical, cylindrical, suboblong or irregular oblate in shape, with a length of 8-65μm and a diameter of 8-25μm, obtuse, obtuse-rounded or relatively flattened at ends, walls of which thickened or relatively thickened, sometimes with slightly sparse, distinct pit canals or slender, oblique pits. The last one is slender, fibrous stone cell, often curved, arranged compactly in bundles, mostly broken, and the intact cell is up to 900μm in length and 10-15μm in diameter, obtuse, round-obtuse or truncated at ends, with walls thickened or relatively thickened, relatively dense pits and pit canals, containing blackish-brown substances in lumen. The laticiferous tubes are mostly branched and colorless or brownish-yellow, 20-50μm in diameter. The parenchyma cells of endopleura, endosperm and cotyledons all contain a number of fine clusters of calcium oxalate, 7-20μm in diameter. Oil droplets and aleurone grains exist in the cells of endosperm and cotyledons. Spiral vessels are slender, 8-18μm in diameter. (Fig.4)

TLC identification

The TLC chromatograms of solutions of samples and reference crude drug present the same color spots at corresponding places. (Fig.5)

Quantitative analysis

Moisture content: The results sho*w* that the weight loss on drying of 10 samples are respectively 6.9%,7.0%, 7.3%, 7.5%, 7.3%, 6.7%, 7.7%, 7.4%, 7.5% and 6.3%.Therefore, the moisture content of *Jatrophae curcatis semen* should be not more than 8.0%.

Alcohol soluble extractive value: The results of 10 samples are separately 18.4%, 15.2%, 14.2%, 18.0%, 16.7%, 13.7%, 15.4%, 14.4%, 15.3% and 16.9%.According to these results, the alcohol soluble extractive value of *Jatropha curcatis semen* should be not less than 14.0%.

Fatty oil content: The results of 10 samples are respectively 39.8%, 40.3%, 42.1%, 37.6%, 40.5%, 41.5%, 36.7%, 39.0%, 45.3% and 42.7%.Hence, the content of fatty oil of *Jatrophae curcatis semen* should be not less than 35.0%.

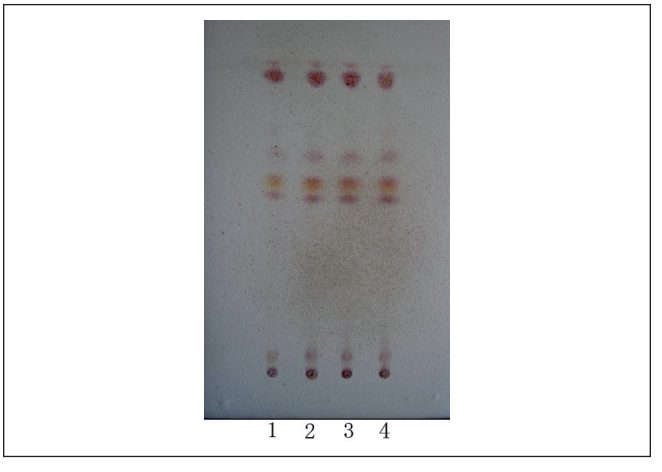

Figure 5: TLC of *Jatrophae curcatis semen*
1-3. test samples; 4. reference crude drug of *Jatrophae curcatis semen*

DISCUSSION

Regarding the microscopic identification, the main microscopical features helpful to correctly identify *Jatrophae curcatis semen* are the appearances of palisade sclereids, pillar stone cells, filiform sclereids and their shapes in both the transverse of section and the powder.

As for the chemical constituents of *Jatrophae curcatis semen,* several literatures have reported that the seed contains ursolic acid[8].But using the TLC identification method, when spotting the solution of ursolic acid used as reference substance to develop with sample solutions, there were no same color spots in chromatogram of samples and reference solution of ursolic acid at the corresponding places. The results were same after repeating the experiments. Consequently, only the solution of reference crude drug was used as reference in the TLC identification. In order to further determine ursolic acid, HPLC method was applied. The powder was made into sample solutions, using ursolic acid as reference substance with an isocratic mobile phase of methyl cyanides: 0.1% phosphoric acid (80:20) to elute. The results showed that there was no chromatographic peak at the same retention time with reference standard (Fig.6). After improving the preparative method of sample solution and chromatographic condition repeatedly, the results were still the same, illustrating that the content of ursolic acid is extremely low, hardly to detectet by HPLC.

The seed possesses anti-fungal and insecticide activities, and also used as purgatives. But it has strong toxicity. When people eat 1-2 seeds, they may manifest dizziness, diarrhea, vomiting and epigastrium burning, and more than two are suggested to be life-threatened[9,10]. Therefore, the seeds are forbidden to take orally, only making the extracts into

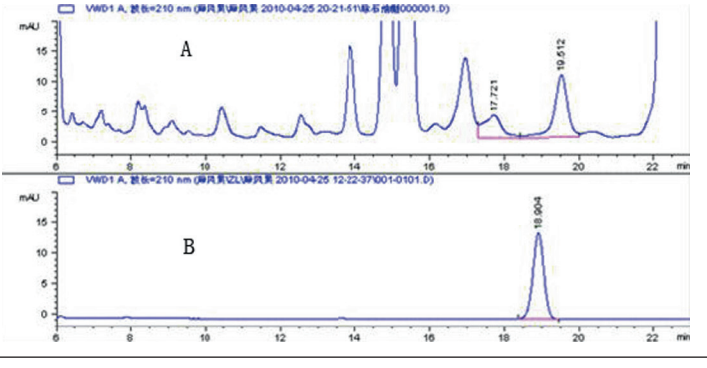

Figure 6: HPLC chromatogram of the extract of *Jatrophae curcatis semen*
A. test samples; B. reference substance of ursolic acid

proper dosage forms to poison *Oncomelania*, *Biomphalaria bulinus* and larva, miracidium, cercaria in the infective stage of *schistosoma*.

CONCLUSIONS

The microscopic characters, the parameters of quantitative analysis and thin layer chromatographic studies can be used for the identification and quality control of the crude drug. This crude drug and their adulterants can be authenticated by using these results. However, these findings are part of a comprehensive study of quality control of *Jatrophae curcatis semen*. The parameters of chemical constituents should be further studied to control the quality.

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Evaluation of pharmacognostic and physicochemical parameters of *Woodfordia fruticosa* Kurz. Flowers

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ABSTRACT

The flowers of *Woodfordia fruticosa* Kurz. (Lythraceae) are reported to have good medicinal values in traditional system of medicines. Present work deals with the pharmacognostical examination of macroscopic and microscopical characters of *Woodfordia fruticosa* Kurz. flowers including detailed anatomy of the pedicel, calyx, pollen grain, anther lobe and ovary. Powder studies showed presence of rosette and cluster crystals of calcium oxalate, annual xylem and unicellular trichome. The physiochemical properties such as loss on drying, total ash value, acid insoluble ash value, water soluble ash value solubility, melting point, pH and extractive values and of flower were carried out. The results of the study could be useful for the identification and preparation of a monograph of the plant.

Keywords: *Woodfordia fruticosa* flower, Pharmacognostic, Physicochemical properties

INTRODUCTION

The use of herbal medicines continues to expand rapidly across the world. Many people now take herbal medicines or herbal products for their health care in different national health-care settings. According to WHO, 80% of the rural population in developing countries depend on traditional medicines to meet their primary health care needs.[1] Authentication and standardization are prerequisite steps while considering source materials for herbal formulation in any system of medicine.[2]

Woodfordia fruticosa Kurz. belongs to the family Lythraceae, is a much branched beautiful shrub, 1-3 m high. It is the plant of tropical and subtropical regions with a long history of medicinal use. The plant is abundantly present throughout India and also in a majority of the countries of South East and Far East Asia like Malaysia, Indonesia, Sri Lanka, China, Japan and Pakistan as well as Tropical Africa.[3] The original Sanskrit name Agnijwala or Tamra-pushpi appears to be derived from the bright red colour of the flower and the bark. Locally (In Gujarat) it is known as Dhavdi.[4,5]

Pharmacognosy basically deals with the standardization, authentication and study of natural drugs. It is closely involved with allied fields, viz. phytochemistry and toxicological screening of natural products. Much of the research in pharmacognosy has been done in identifying controversial species of plants, authentication of commonly used traditional medicinal plants through morphological, histological, physicochemical and toxicological parameters, especially heavy metal estimation and radiobiological contamination in plants, prescribed by an authoritative source. The importance of pharmacognosy has been widely felt in recent time.[6]

To ensure reproducible quality of herbal medicines, proper control of starting material is utmost essential. The first step towards ensuring quality of starting material is authentication followed by creating numerical values of standards for comparison. Pharmacognostical parameters for easy identification like flower constants, microscopy and physico chemical analyses are few of the basic protocol for standardization of herbals. Hence, in the present work the pharmacognostical standardization has been performed for the flower of the plant.

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MATERIAL AND METHODS

Collection and extraction of plant material

The fresh flowers of *Woodfordia fruticosa* were collected from Junagadh (Girnar region), Gujarat in the month of March 2008. The plant was compared with voucher specimen (voucher specimen No. PSN303) deposited at Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India. The flowers were washed under tap water, air dried, homogenized to fine powder and stored in airtight bottles. Ten grams of dried powder was first defatted with petroleum ether and then extracted with methanol by using Soxhlet apparatus.[7] The solvent was evaporated to dryness and the dried crude extract was stored in air tight bottle at 4°C. The percentage yield of methanol extract was 36%. The methanolic extract was used for the solubility study.

Pharmacognostic studies

Macroscopic characteristics

The plant was macroscopically examined for shape, size, surface characteristics, texture, color, consistency, odour, taste, etc.[8]

Microscopic characteristics

Free hand sections of pedicel, calyx, sepal and ovary of fresh flower of Woodfordia fruticosa were taken. Sections were cleared with chloral hydrate and then stained with phloroglucinol and hydrochloric acid and mounted with glycerin. Same procedure was followed for microscopic characteristics of powdered material of *Woodfordia fruticosa* flower.[8]

Physicochemical parameters

Physicochemical parameters were determined as per guidelines of WHO (2002) [9]. Total ash value, loss on drying, water soluble ash, acid insoluble ash, solubility, melting point, pH, analysis, petroleum ether soluble extractive, alcohol soluble extractive value and water soluble extractive value were determined.

RESULTS

Macroscopic characteristics

The full-grown *Woodfordia fruticosa* shrub is about 3.0 m high, having long and spreading branches with fluted stems. The flowers are bright red, innumerable, arranged in dense axillary paniculate-cymose clusters (Figure 1), with short glandular pubescent pedicels. The inflorescence is deep red, 2-15 flowered, fascicled cymes. The calyx is long (11-33 mm), striated, covered with glandular dots, with a small campanulate base and a long slightly curved bright red tube. The petals are pink, papery, slightly longer than the calyx-teeth, narrowly linear, extended at the apex to a long fine point. The fruits are small capsules, ellipsoid and membranous, usually splitting the calyx near the base, and are irregularly dehiscent. The seeds are brown, numerous, very minute, smooth, shining, angular and obovate.

Microscopic characteristics

Pedicel

A transverse section of the pedicel flower shows a single-layered epidermis, with a fairly thick cuticle. Numerous unicellular trichomes arise from this layer. The epidermis is followed by a 7-8 layered cortex, differentiated into collenchyma and parenchyma with plenty of air spaces. The primary xylem is represented by uni- or bi-seriate groups

Figure 1: Macroscopic characteristics of *Woodfordia fruticosa*

of 3 or 4 tracheids arranged in a ring with phloem on either side of the xylem. The rosette and cluster crystals of calcium oxalate are found in the cortex (Figure 2).

Calyx

A transverse section of the calyx tube is circular in outline. The cells of the upper epidermis in surface showed scattered trichomes. The calyx tube consists of several layers of ground tissue containing rosettes and cluster of calcium oxalate crystals and bounded on either side by upper and lower epidermis respectively. Anomocytic, actinocytic and anisocytic stomata are present. Vascular bundles are small, collateral and surrounded by bundle sheath. In sepals, the cells of the lower epidermis in surface view are broad, slightly irregular, thin walled in the upper region but thick walled in the basal region of the calyx. The tissue is differentiated into an adaxial palisade and an abaxial spongy parenchyma in the upper ¾ of the calyx tube (Figure 2).

Anther lobes

The anther lobes are tetrasporangiate and the walls separating the locules get disorganized. A transaction of a lobe shows an epidermis formed of large colourless cells followed by a fibrous layer, which appears crinkled (Figure 3).

Pollen grains

Pollen grains are 3-zonocolporate, oblate spheroidal shape and it's surface is psilate (Figure 3).

TS of ovary

The ovary is bicarpellary and laterally flattened and as such appears elongated in transaction (Figure 3).

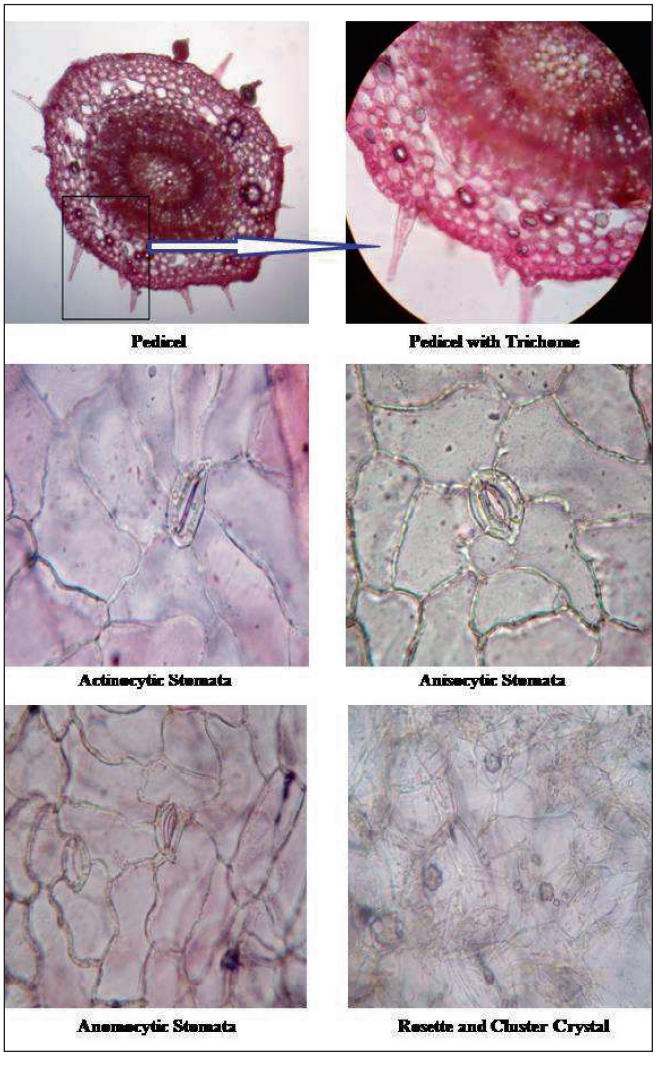

Figure 2: Microscopic characteristics of *Woodfordia fruticosa* flower

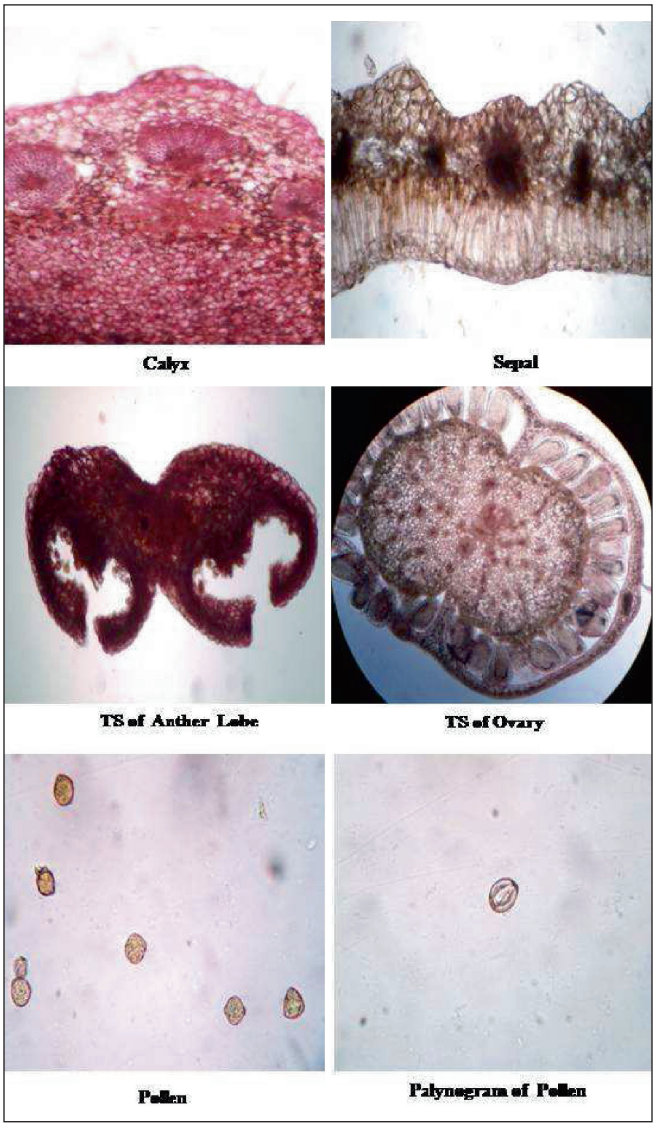

Figure 3: Microscopic characteristics of *Woodfordia fruticosa* flower

Powder characteristic

The crude powder of *Woodfordia fruticosa* flower was light brown in color, slightly bitter and astringent in taste. Microscopy study of powder showed the presence of epidermis in surface view showed straight wall cells. Few cells of fibrous layer observed in powder. Cluster crystals of calcium oxalate were present in powder. Pollen grains either singly or in groups were found. Simple covering unicellular trichomes are present. Annular xylem vessels were found (Figure 4).

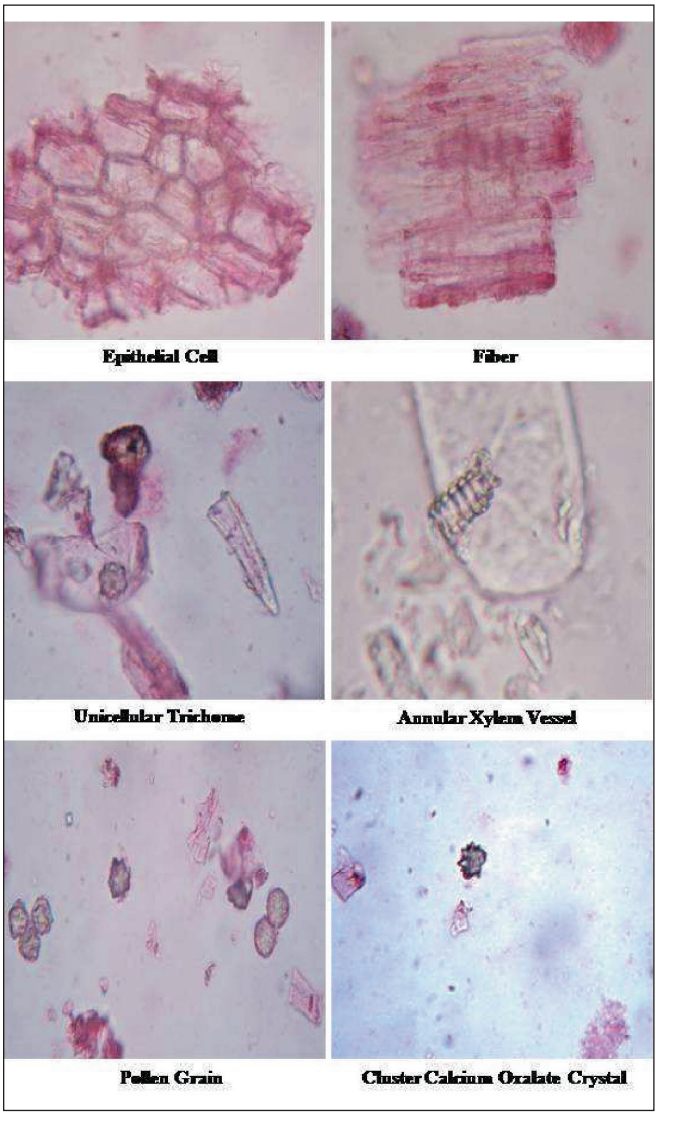

Figure 4: Powder characteristics of *Woodfordia fruticosa* flower

PHYSICOCHEMICAL ANALYSIS

Proximate parameters analysis

The result of proximate analysis of crude powder of *Woodfordia fruticosa* flower is shown in Table 1. The average values are expressed as percentage of air-dried material. The loss on drying was 8%. Total ash was 5.45%, acid insoluble ash was 0.57% and water soluble ash was 2.47%. The extractive value of crude powder was maximum in water (41.59%), followed by methanol (32.77%) and minimum was in hexane (0.71%). pH and melting point of methanol extract was 3.5 and 114°C respectively.

Solubility test

The methanol extract of *Woodfordia fruticosa* flowers was evaluated for solubility in 10 solvents with varied polarities. The extract was highly soluble in dimethylformamide, methanol and dimethylsulphoxide but insoluble in hexane, petroleum ether and toluene solvents (Table 2).

Table 1: Determination of proximate parameters of crude powder of Woodfordia fruticosa flowers

Parameters	Value (w/w)
Loss on drying	8%
Total ash	5.45%
Acid insoluble ash	0.57%
Water soluble ash	2.47%
Petroleum ether soluble extractive	0.74%
Hexane soluble extractive	0.71%
Ethyl acetate soluble extractive	1.80%
Acetone soluble extractive	8.08%
Methanol soluble extractive	32.77%
Water soluble extractive	41.59%
pH of methanol extract	3.5
Melting point methanol extract	114°C

Table: 2 Determination of solubility of methanol extract of Woodfordia fruticosa flowers in different solvents.

Solvent	Solubility (mg/ml)
Acetone	6.0
Chloroform	3.0
Dimethylformamide (DMF)	119.0
Dimethylsulphoxide (DMSO)	109.8
Distilled water	34.2
Ethyl acetate	3.0
Hexane	-
Methanol	113.1
Petroleum ether	-
Toluene	-

DISCUSSION

The pharmacognostical study is a major and reliable criterion of identification of plant drugs. The pharmacognostic parameters are necessary for confirmation of the identity and determination of quality and purity of a crude drugs.[10] To ensure reproducible quality of herbal products, proper control of starting material is utmost essential.[11] Thus, in recent years there has been an emphasis on standardization of medicinal plants, and evaluation of plant drugs by pharmacognostical studies is still more reliable, accurate and inexpensive means. Pharmacognostic studies on different plants has been done by various workers.[12,13,14,15,16]. According to World Health Organization (WHO) the macroscopic and microscopic description of a medicinal plant is the first step towards establishing its identity and purity and should be carried out before any tests are undertaken.[9]

The flowers of *Woodfordia fruticosa* have three types of matured stomata viz. anomocytic, actinocytic and anisocytic. Stomata is the main factor responsible for the physiological activities of the plant, abnormal stomata is responsible for behavior and hormonal imbalance in plants.[17] In calyx both rosette and cluster type of calcium oxalate crystals were found; these could be used to distinguish the species.

The physical constant evaluation of the powder is an important parameter in detecting adulteration or improper handling of drugs. The percentage of active chemical constituents in crude drugs is mentioned on air-dried basis. Therefore, the loss on drying of plant materials should be determined and the water content should also be controlled. The moisture content of dry powder of *Woodfordia fruticosa* flowers was 8 % which is not very high, hence it would discourage bacteria fungi or yeast growth.[10] The total ash is particularly important in the evaluation of purity of drugs, i.e. the presence or absence of foreign inorganic matter such as metallic salts and/or silica.[18,19] Low amount of total ash, acid insoluble ash and water soluble ash indicate that the inorganic matter and non-physiological matter such as silica is less in *Woodfordia fruticosa* flowers. The extractive values are useful to evaluate the chemical constituents present in the crude drug and also help in estimation of specific constituents soluble in a particular solvent. The variation in extractable matter in various solvents is suggestive of the fact that the formation of the bioactive principle of the medicinal plants is influenced by number of intrinsic and extrinsic factors. High alcohol soluble and water soluble extractive values reveal the presence of polar substance like phenols, tannins and glycosides, as reported by Sharma et al. (2009).[20]

CONCLUSIONS

The present study may be useful to supplement information in respect to its identification, authentication and standardization of herbal drugs. In other words, the pharmacognostic features examined in the present study may serve as tool for identification of the plant for validation of the raw material and for standardization of its formulations at herbal industrial level in the coming days.

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Pharmacognostical standardization of leaves of *Alangium lamarckii* Thwaites

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ABSTRACT

Alangium lamarckii Thwaites is traditionally found to be useful for many ailments. The present study highlights the pharmacognostical as well as phytochemical studies including parameters such as macroscopic, microscopic characters, physiochemical evaluation and preliminary phytochemical studies of the leaves. Morphological studies revealed leaves were varying in length and breadth from 7 to12 cm in length and 2 to 5 cm in width. Some diagnostic characters are presence of unicellular covering trichrome varying in length from 54 µm to 150 µm, presence of branched sclerides and prism of calcium oxalate varying in size from 11x 7µm to 18x 11µm. The various physico-chemical standards and their percentage w/w were found to be: total ash (7.62%), acid insoluble ash (1.66%), water soluble ash (2.11%), alcohol soluble extractive value (18.24%), water soluble extractive value (27.36%) and loss on drying (13.01%). Preliminary phytochemical investigation showed the presence of alkaloids, phenols, tannins, triterpenoids, phytosterols, amino acids and carbohydrates. Total phenolic content by Folin-Ciocalteu method is found to be 150.62 mg/g equivalent to gallic acid. Total alkaloid content quantified by gravimetric method was found to be 1.114% w/w. TLC studies on leaf of *A. lamarckii* has been carried out. These observations will help in identification and standardization of the drug in the crude form and also to distinguish the drug from its adulterants.

Key words: *Alangium lamarckii*, quantitative standards, TLC profile, total phenol, total alkaloid

INTRODUCTION

Alangium lamarckii Thwaites (Syn. *A. salviifolium*) belongs to family Alangiaceae is found commonly in Tropical forest of South India and occasionally grown in garden. The root, root bark, seeds and leaves of the plant is used in Indian system of medicine. The root and root bark of the plant are used as antihelmenthic and purgative, whereas fruits are used as cooling, nutritive and tonic. Leaves are useful for curing diabetes[1-2] Decoction of bark has been used as an emetic in India.[3] Methanol extract of *A. salviifolium* flowers shown to have antibacterial activity against both gram-positive and gram-negative bacteria.[4] Methanolic extract of root of *A. salviifolium* have shown to possess analgesic and anti-inflammatory activities in albino mice.[5] The lyophilized powder extract of pulverized wood of *A. salviifolium* showed significant antifungal activity against *Candida albicans*.[6] Ethanolic extract of leaves of *Alangium salviifolium* have showed wound healing potential in rats by incision, excision, dead space (granulation) wound models at two different doses (150 and 300 mg/kg).[7] Alangium A and B from root bark and akoline, lamarkine, alangine, akharkantine from bark, have been reported.[8] The present investigation on leaf of *A. lamarckii* is therefore taken up to establish certain pharmacognostical and chemical standards which would help in identification as well as in checking adulteration, if any, further the study will greatly help in quality assurance of finished products of herbal drugs.

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MATERIALS AND METHODS

The leaves of *Alangium lamarckii* Thwaites were collected from Panakudi, Tirunelveli District in the month of July 2009 and identified and authenticated by Prof. V. Chelladurai, Chief Research Botanist (Retd.) Palayamkottai, Tamilnadu, India. For future reference the voucher specimen (Specimen number-COG/AL/09) and the prepared herbarium was

deposited at the Department of Pharmaceutics, Banaras Hindu University, Varanasi (U.P), India.

Morphological (i.e. macroscopical) characters of the plant material was studied on the basis of sensory characteristics viz. size, shape, colour, odour, texture of plant part use as drugs. Numerous free hand sections were taken, stained and mounted following the usual microtectnique described by Johansen[9] and photographs of different magnifications were taken with Nikon (Eclipse E200) microscopic units. Toludine blue is used to stain the sections. Since toluidine blue is a polychromatic stain, the staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink color to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. For the study of isolated cells leaves were macerated with concentrated nitric acid and potassium chlorate, washed with distilled water and mounted in glycerin. For quantative microscopy of leaves i.e. palisade ratio, vein-islet and vein let termination number, stomatal number and stomatal index, leaves were boiled with 1% chloral hydrate solution. The upper and lower epidermis was peeled out separately by means of forceps. The mount of cleared leaf and upper and lower epidermis and were separately prepared in diluted glycerin. The value of quantative microscopy was determined as per the method described by Wallis.[10] Ultraviolet fluorescence analysis of powdered drug was done as per the method described by Kokoski et al & Chase and Pratt.[11-12] Leaves powder was subjected to preliminary and microscopical examination, physiochemical evaluations which include ash value, acid insoluble and water soluble ash, extractive value (water soluble and ethanol soluble) and moisture contents (loss on drying) as per the method given in Indian pharmacopoeia 1996.[13]

Preliminary phytochemical screening of alcoholic and aqueous extract was done by method described by Khandelwal[14] and Harbone.[15] Total phenolic (TP) content of crude alcoholic extract was determined by Singleton et al.[16] The folin-Ciocalteu (FC) assay was carried out by pipetting 1 ml of extract into test tube and to it 8 ml water was added. To this 0.5 ml of FC reagent was added. The mixture was vortexed for 30s and 1.5 ml of filtered 20% sodium carbonate solution was added after 15 min. The absorbance of the coloured reaction product was measured at 765 nm after 2h at ambient temperature. A calibration curve was created using different concentrations of standard gallic acid solutions. The level of TP in the extract was calculated from the standard calibration curve. Results were expressed on the basis of mg of Gallic Acid Equivalent per gram (mg GAE/g) of dried ethanolic extract. The total tannin content was measured according to the methods described by Grubesic et al.[17] The estimation of the total alkaloid content was done by gravimetric methods.[18] Thin layer chromatography, and developing of chromatogram was done as per the methods proposed by Wagner.[19] The pre-coated aluminum silica gel plates 60 F_{254} were used. The solvent systems used for development of chromatogram were the mixtures of different polarity. Spraying reagent used for identification were vanillin-sulphuric acid, reagent (for triterpenes and steroids), Dragandroff's reagent for alkaloids and sodium metaperiodate and benzidine for sugars and glycosides.

RESULTS

Morphological study

Morphological studies of dorsiventral leaf of *A. lamarckii* is dark green in colour on adaxial side and light green on abaxial side, bitter in taste, odorless, leaves are vary in length and breadth from 7 to12 cm in length and 2 to 5 cm in width. Leaf is simple with short petiole, petiole 6 to13 mm long, densely pubescent, lanceolate in shape, entire margin, venation prominent on abaxial side and flat on adaxial side, venation reticulate, apex acute and surface smooth (Plate1).

Microscopical features

The microscopic feature of the leaf of *A. lamarckii* was presented in the Plate1. Leaf has thick lamina and fairly prominent midrib. The midrib is broadly hemispherical on the abaxial side and shallow convexity on the adaxial side. It is nearly 760 µm in vertical plane. The adaxial and abaxial epidermal layers of the mid rib region are thin, made up of squarish, thick walled, heavily cuticularized cells. Unicellular unbranched trichomes, present on adaxial sides of the midrib. The epidermis is followed by two to four layers of collenchymatous cells with an angular thickening on the both sides of midrib. The ground tissue is made up of five to six layers of parenchymatous cells without intercellular space. Vascular bundle is surrounded by sclerenchymatous tissue except on lateral sides. The vascular strand of midrib is single, large and prominent. The xylem elements are thick walled, angular and compactly arranged in parallel rows. Phloem elements are in small nests of five to ten cells. The ground parenchyma cells contain prism of calcium oxalate crystals, varying in size from 11x 7µm to 18x 11µm.

The microscopic features of the lamina of *A. lamarckii* are presented in Plate1. Lamina is 204 µm thick. The adaxial epidermis is single layered; 24 µm thick. The epidermal

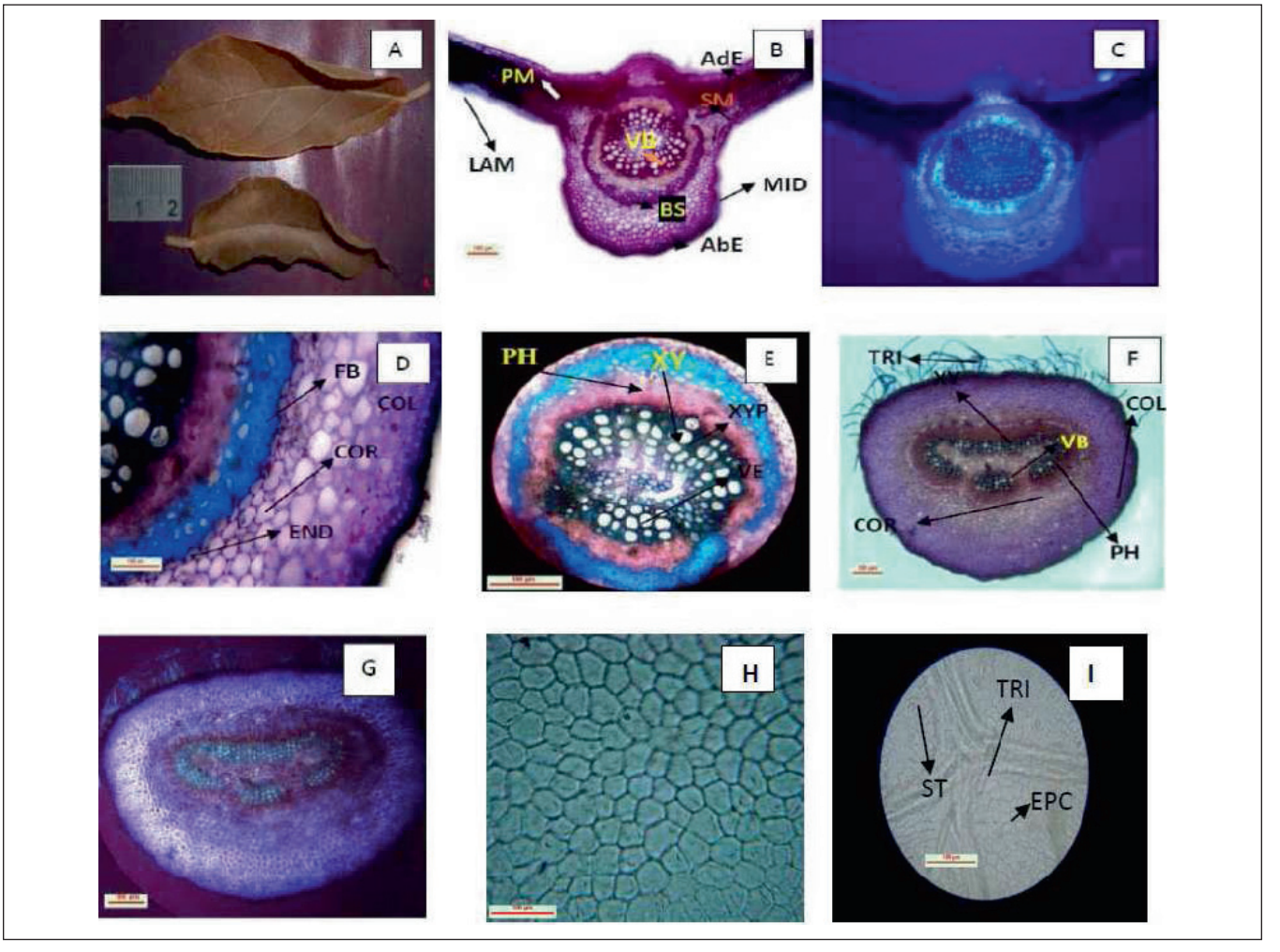

Plate 1: Figs.A-I. Macro- and Microscopical character of the leaves of *A. lamarckii*
A. Leaf; B. TS of mid rib with lamina; C. TS of mid rib with lamina in polarized light; D. region of mid rib from epidermis to vascular bundle enlarged; E. Vascular bundle of mid rib enlarged; F. TS of petiole; G. TS of petiole in polarized light; H. Adaxial epidermis in high magnification (40X); I. Abaxial epidermis in high magnification (40X).
(Abbreviation: AdE-adaxial epidermis; AbE-abaxial epidermis; LAM-lamina; MID-midrib; PM-palisade mesophyll; SM-spongy mesophyll; BS-bundle sheath; VB-vascular bundle; COL-collenchyma; COR- cortex; END-endodermis; FB-Fiber; PH-Phloem; XY-xylem; XYP-xylem parenchyma; VE-vessels; TRI-trichrome; ST-stomata; EPC-epidermal cell

cells of the lamina are rectangular shaped with outer convex wall and thin cuticle. The abaxial epidermis is comparatively thin with cells of varying shape and size. The abaxial side of epidermis showing presence unicellular covering trichrome, varying in size from 54 μm to 340 μm. The mesophyll tissue is differentiated into adaxial palisade zone and abaxial spongy parenchyma zone. Palisade is double layered in lamina and become single layered towards the margin region, cylindrical, compact and occupies one-third thickness of lamina. The spongy parenchyma cells are lobed and loosely arranged. The vascular strands of lateral veins are situated in the spongy mesophyll, just beneath the palisade zone. The lateral veins have small collateral vascular bundle with parenchymatous bundle sheath, adaxial and abaxial extensions.

The adaxial epidermis is apostomatic i.e. devoid of stomata. The cells are polygonal with straight, thin, anticlinal walls. Abaxial epidemis is stomatiferous; the stomata are mostly anomocytic. The epidermal cells are large, polygonal with straight or slightly wavy thin anticlinal walls. The lateral veins are fairly thick; the vein lets are thin and profusely branched. The vein islets are distinct, wide and variable in outline. The vein terminations are also distinct, wide and variable in outline. The vein terminations are also distinct. They are either simple or branched, twice or thrice (Plate1).

Petiole is nearly 1292 μm in vertical plane. Epidermis single layered, covered by cuticle. Adaxial surface of petiole have presence of abundant unicellular covering trichrome. Size

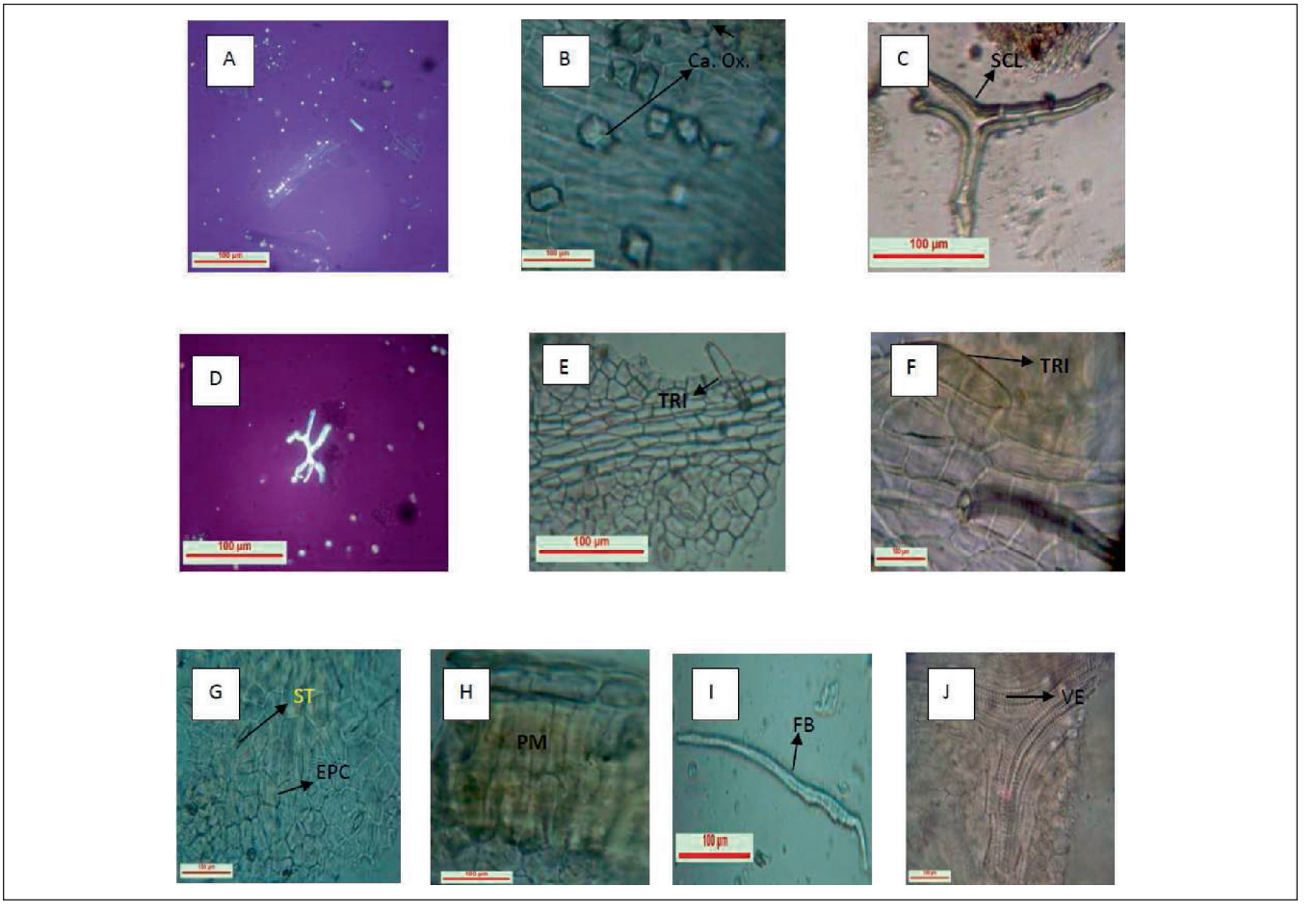

Plate 2: Figs. A-J. Powder characteristics of leaves of *A. lamarckii*
A. Calcium oxalate crystals in polarized light; B. Calcium oxalate crystals in ordinary light; C. Sclerids in ordinary light; D. Sclerids in polarized light; E-F. Fragments of tissue showing unicellular covering trichrome; G. Fragments of tissue showing presence of anomocytic stomata; H. Fragments of tissue showing mesophyll cells; I. Fibres; J. Fragments of tissue showing presence of vessels.
(Abbreviation: Ca. Ox.-Calcium oxalate crystals; SCL-sclerids; TRI- trichrome; EPC- epidermal cells; ST- stomata; PM-palisade mesophyll; FB-fibers; VE-vessels).

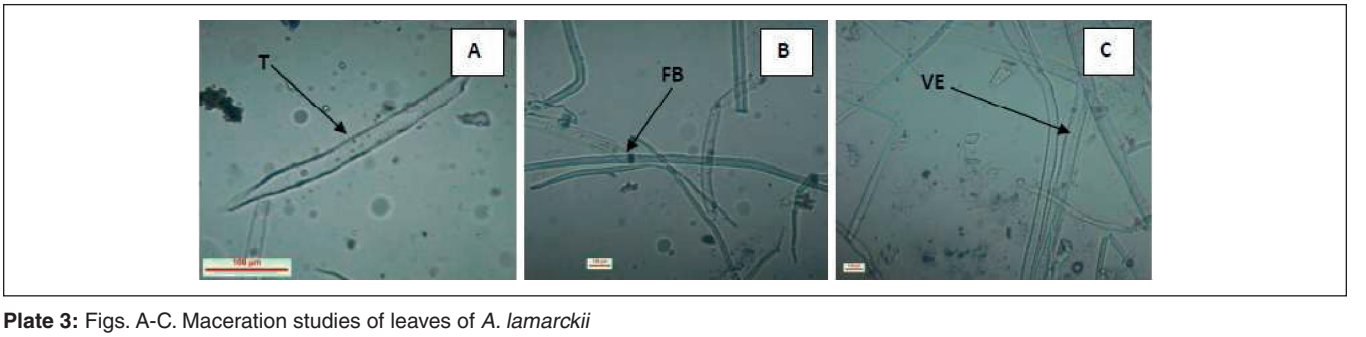

Plate 3: Figs. A-C. Maceration studies of leaves of *A. lamarckii*
A. Tracheid in high magnification (40X); B. Fibers in low magnification (10X); C. Vessels in low magnification.
(Abbreviation: T-Tracheid; FB-fibers; VE- vessels).

of the trichrome varies from 70 µm to 300 µm. Cortex is composed of collenchymatous cells, 6 to10 layered present just beneath the epidermis, followed by parenchymatous tissue; collateral vascular bundles are arranged in arch and surrounding parenchymatous tissue. (Plate1).

Powdered plant material

Powder as such

The powdered leaf is greenish brown in colour, characteristic odour and bitter in taste.

Powder mounted on slide with various reagents i.e. powder microscopy (Plate2).

Powder shows presence of lignified branched sclerides, tracheids, vessels with spiral thickening, fibers with tapered ends, unicellular covering trichrome, groups of palisade cells, fragments of upper epidermis and lower epidermis with anomocytic stomata.

Maceration study of leaf

Maceration study of leaf shows the presence of tracheids, vessels with spiral thickening and fibers (Plate 3).

Quantitative microscopy

The palisade ratio, stomatal number, stomatal index, vein islet number and vein termination number of leaves are given in Table1.

Physiochemical constants

Determination of physiochemical constants were carried out and their result was presented in Table 2.

Fluorescence analysis

Fluorescence analysis of leaf powder was studied and observations were shown in table 3.

Preliminary phytochemical screening

Preliminary phytochemical screening of alcoholic and aqueous extract was carried to show the presence of various phytoconstituents (Table 4).

Total phenolic and tannin content

Total phenol and tannin content of alcoholic extract obtained by cold maceration of powdered leaves were found to be 150.62 mg/g and 77.63 mg/g of dry extract equivalent to gallic acid respectively.

Total alkaloid content

Total alkaloid content quantified by gravimetric method was found to be 1.114% w/w.

Table 1: Leaf constant of *Alangium lamarckii*

Parameters	Value
Stomatal number	
Adaxial epidermis	-
Abaxial epidermis	16-24/mm2
Stomatal Index	
Adaxial epidermis	-
Abaxial epidermis	9.8-19.1
Vein termination number	26.3-38.2/mm2
Vein islet number	10.2-13.5mm/2
Palisade ratio	3.2-7.5

Table 2: Physico-chemical constants*

1. Ash value	
Total ash	7.62% w/w
Water soluble ash	2.11% w/w
Acid insoluble ash	1.66% w/w
2. Extractive value	
Alcohol soluble extractive	18.24% w/w
Water soluble extractive	27.36% w/w
3. Loss on drying	13.01% w/w

* Mean value of three individual's readings

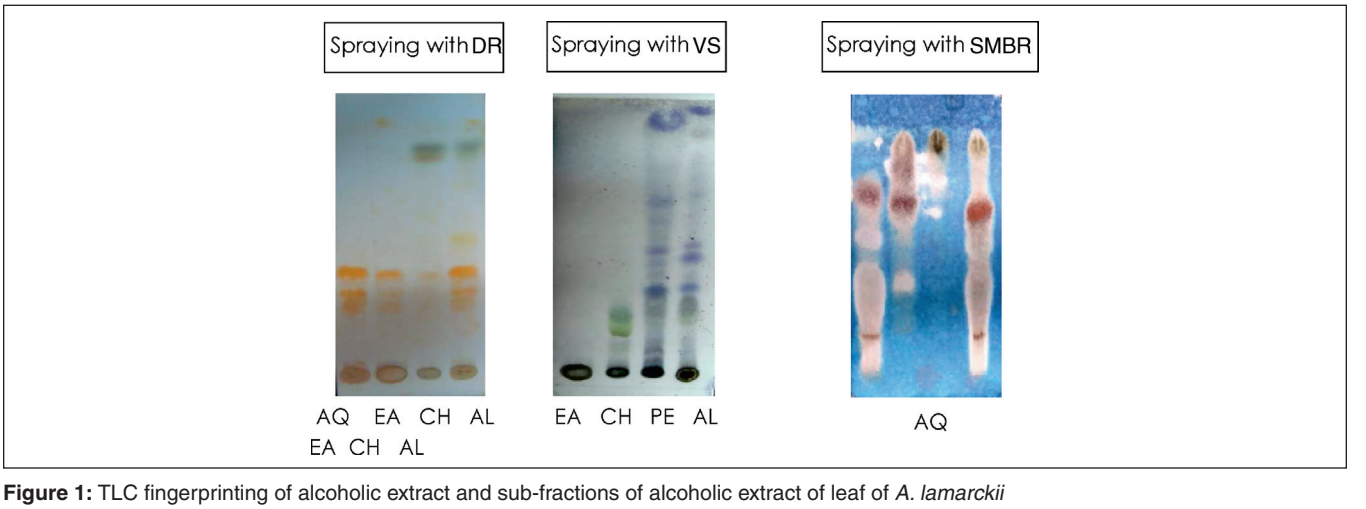

Figure 1: TLC fingerprinting of alcoholic extract and sub-fractions of alcoholic extract of leaf of *A. lamarckii*
(Abbreviation: DR- Dragendroff's reagent; VS- Vanillin sulphuric acid; SMBR- Sodium metaperiodate followed by benzidine reagent AL-Alcoholic extract; PE-Petroleum ether fraction; CH-Chloroform fraction; EA-Ethyl acetate fraction; AQ-Aqueous fraction)

Table 3: Fluorescence analysis of leaf of *A. lamarckii*

S. No.	Mixture + Powder	Fluorescence in day light	Fluorescence in U.V. light
1.	Powder as such	Dark brown	No fluorescence (N.F.)
2.	Powder + 1N NaOH in methanol	Olive	Sandy brown
3.	Powder + 1N NaOH in water	Fire brick	Yellow green
4.	Powder + 1N HCl in methanol	Golden rod	Medium green
5.	Powder + 1N HCl in water	Dark green	Sandy brown
6.	Powder + 1N HNO3 in methanol	Dark green	Orange
7.	Powder + 1N HNO3 in water	Golden rod	Pale green
8.	Powder + 5% Iodine	Red	N.F.
9.	Powder + 5% FeCl3	Black	N.F.
10.	Powder + 50% KOH	Brown	Yellow green
11.	Powder + NH3	Brown	Green yellow
12.	Powder + Picric acid	Yellow	N.F.
13.	Powder + Acetic acid	Dark green	Sandy brown

Table 4: Preliminary Phytochemical screening of *A. lamarckii*

S. No.	Test	Alcoholic Ext.	Aqueous Ext.
1.	Phenols		
	Ferric chloride test	+ + +	+ + +
2.	Alkaloids		
	a. Mayer's test	+ + +	+ + +
	b. Dragendorff's test	+ + +	+ + +
	c. Wagner's test	+ + +	+ + +
	d. Hager's test	+ + +	+ + +
3.	Flavonoids		
	Shinoda test	- - -	- - -
4.	Saponins		
	Foam test	- - -	- - -
5.	Tannin		
	Lead acetate test	+ + +	+ + +
6.	Triterpenoides		
	Thionyl chloride test	+ + +	- - -
7.	Phytosterols		
	a. Liberman Burchard test	+ + +	- - -
	b. Salkowski test	+ + +	- - -
8.	Carbohydrates		
	a. Molish's test	+ + +	+ + +
	b. Reducing suger test	+ + +	+ + +
9.	Amino acids		
	Ninhydrine test	+ + +	+ + +
10.	Glycosides	- - -	- - -
	a. Legal's test	- - -	- - -
	b. Borntrager's test	- - -	- - -
	c. Modified Borntrager's test		

+ + + Indicates present
- - - Indicates absent

Thin Layer Chromatography (TLC)

Thin layer chromatography of alcoholic extract and their various sub-fractions was done on pre-coated aluminum silica gel plates 60 F_{254} as stationary phase and using different solvent systems and sprayed with Dragendroff's reagent, to show presence of alkaloids, vanillin sulphuric acid for the presence of terpenoidal molecules and sodium metaperiodate followed by benzidine to shows the presence of sugars or glycosides (Fig.1). The results of TLC fingerprinting of alcoholic extract and their sub-fractions were summarized in table 5.

DISCUSSION

The evaluation of a crude drug is an integral part of establishing its correct identity. Before any crude drug can be included in herbal pharmacopoeia, pharmacognostical parameters and standards must be established. Therefore some diagnostic features have been evolved to identify and to differentiate the *A. lamarckii* leaf and from the other crude drugs and its adulterants. In this regard the important microscopic features of the leaf has been documented such as presence of unicellular covering trichrome, branched sclerides and presence of calcium oxalate crystals. The results obtained in pharmacognostical studies reported herein established the macro and microscopic parameters that characterize the genuine plant drug *A. lamarckii*. These characteristics can be utilized for quick identification of the drug and are particularly useful in the case of powdered materials. In quantitative microscopy, the stomatal index was found to be 9.8-19.1. Vein islet number and vein-let termination number are 10.2-13.5mm/^{2} and 26.3-38.2/mm^2 respectively which can help to identify the closely related species of *Alangium* genus. Preliminary phytochemical investigation showed the presence of alkaloids, phenols, tannins, triterpenoids, phytosterols, amino acids and carbohydrates. The total ash of leaf was found to be 7.62% w/w, of which, acid insoluble ash was 1.66% w/w and water soluble ash was 2.11% w/w. The extractive values were found to be 18.24% w/w alcohol and 27.36% w/w water respectively. Studies on physico-chemical constants can serve as a valuable source of information and provide suitable standards to determine the quality of this plant. TLC studies of alcoholic extract and there sub-fractions justified the presence of alkaloids, terpenoides and glycosides or sugers. The parameters, which are being reported first time in this work, could be useful in the preparation of the herbal monograph for its proper identification.

ACKNOWLEDGEMENT

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Table 5: TLC pattern of alcoholic extract and sub-fractions of *A. lamarckii*

Extract & sub-fractions	Solvent system	Spraying reagents	No. of spot	R_f value
Alcoholic	Toluene:ethyl acetate: diethylamine (70:20:10)	Dragendroff's reagent	4	0.26, 0.30, 0.33 and 0.41
Chloroform			1	0.33
Ethyl acetate			3	0.26, 0.30 and 0.33
Aqueous			3	0.26, 0.30 and 0.33
Alcoholic	Toluene:ethyl acetate (93:7)	Vanillin-sulphuric acid	4	0.99, 0.55, 0.50 and 0.41
Petroleum ether			5	0.99, 0.71, 0.55, 0.50 and 0.41
Chloroform			nil	-
Ethyl acetate			nil	-
Alcoholic	Butanol: acetic acid: water (5:1:4)	Sodium metaperiodate followed by benzidine	4	0.51, 0.60, 0.68 and 0.91
Chloroform			nil	-
Ethyl acetate			1	0.51
Aqueous			4	0.51, 0.60, 0.68 and 0.91

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Establishment of Quality Parameters of Roots and Rhizomes of *Oxystelma esculentum*

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ABSTRACT

Introduction: *Oxystelma esculentum* is a perennial twiner growing near water-logged areas. Its roots are traditionally used as diuretic, galactagogue, anthelmintic, antiulcer, laxative and antiperiodic. The roots are also used ethnomedicinally by the tribes of Orissa in India for treating hepatitis. **Methods:** The present investigation deals with the pharmacognostic study of the roots and rhizomes of *Oxystelma esculentum* and establishment of its quality parameters, including physicochemical and phytochemical evaluation. **Results:** It was found that the roots and rhizomes of *O. esculentum* have almost similar microscopic features. They show the presence of cork and occasional lenticels, followed by phelloderm and cortex consisting of triangular or oval stone cells having U-shaped lumen. The pericycle consists of 4-5 continuous bands of stone cells followed by stele having endarch xylem. Sheath of calcium oxalate rosette crystals is present above the xylem. Pith is present in rhizome but absent in root. Microscopy of the powder revealed the presence of cork in surface view, stone cells, parenchymatous tissue lined internally by a large number of calcium oxalate rosette crystals and xylem vessels with different types of thickening. Various physico-chemical parameters and quantitative microscopic parameters were established. From the phytochemical screening, the roots and rhizomes were found to contain cardenolides, flavonoids, phenolics and sugars, which were estimated by their respective procedures. **Conclusion:** Establishment of these quality parameters can be useful in the identification, authentication and standardization of the plant material while also paving a way for exploring its phytoconstituents and possible therapeutic applications.

Key words: *Oxystelma esculentum*, Jaldudhi, Dudhlata, Asclepiadaceae.

INTRODUCTION

Oxystelma esculentum R. Br. syn. *Oxystelma secamone*, *Periploca esculenta*, *Periploca secamone*, *Sarcostemma secamone, Sarcostemma esculentum* and *Asclepias rosea* (Family – Asclepiadaceae) is commonly known as 'Jaldudhi' or 'Dudhlata' and is found near water logged areas of the plains and lower hills of the Indian subcontinent and Java.[1] The roots have been reported to possess antiperiodic, anthelmintic, diuretic, laxative, antiulcer and galactagogue activity.[2] They are used ethnomedicinally by the tribes of Orissa in India in throat infections, skin diseases and also in the treatment of hepatitis.[3] The present study deals with the pharmacognostic study of the roots and rhizomes of the plant and establishment of its various quality parameters.

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MATERIALS AND METHODS

Collection and authentication of the plant

O. esculentum was collected in the flowering and fruiting stage from Barda Hills, Gujarat, India in November, 2008. Voucher specimen (No. RKCP/COG/01/2008) was deposited in R K College of Pharmacy, Rajkot. Authentication of herbarium was done by Dr. N. R. Sheth, Head of Department of Pharmaceutical Sciences, Saurashtra University.

Pharmacognostic studies

Fresh roots and rhizomes were used for pharmacognostical studies and quantitative microscopy. The roots and rhizomes were dried under shade and powdered to 60# separately and stored in airtight containers and used for physico-chemical evaluation and phytochemical studies. Macroscopical and

microscopical studies of the roots and rhizomes were performed. For microscopical studies, chloral hydrate was used as clearing agent and phloroglucinol with conc. HCl was used for staining. Photomicrography of the transverse sections and the powdered drug was performed using light microscope (Labomed) and WinDVR camera and software. Quantitative microscopic study was performed using camera lucida and stage micrometer scale (Table 1).[4]

Table 1: Quantitative microscopy

Parameters	Measured values (µ)
Xylem vessel length	120.3 – 201.77 – 259.89
Xylem vessel diameter	92.23 – 109.24 – 119.27
Stone cell diameter	52.48 – 63.31 – 77.15
Rosette crystal diameter	12.88 – 23.39 – 37.2

Number of observations = 50

Table 2: Physico-chemical evaluation

Parameters	% w/w± SD
Loss on drying	70.20 ± 0.50
Ash values	
Total ash	10.20 ± 0.25
Acid insoluble ash	0.75 ± 0.20
Water soluble ash	6.15 ± 0.20
Extractive values	
Water soluble extractive	4.1 ± 0.18
Alcohol soluble extractive	2.2 ± 0.16

Number of observations = 5
SD = Standard Deviation

Physico-chemical evaluation

This included determination of moisture content, ash values (total ash, acid insoluble ash and water soluble ash) and extractive values (water and alcohol soluble extractives) (Table 2).[5]

Phytochemical studies

Phytochemical screening was performed (Table 3).[6-11] Estimation of phytoconstituents included that of cardenolides,[12] phenolics,[13] flavonoids[14] and sugars[15] (Table 4).

RESULTS AND DISCUSSION

Pharmacognostic study

Macroscopy

Roots and rhizomes are rough, creamish brown, cylindrical, 5-8mm wide, having wiry lateral roots, lenticels and longitudinal striations. No characteristic odor or taste is present (Figure 1).

Microscopy: Transverse section

Roots & rhizomes of *O. esculentum* have almost similar microscopic features (Figure 2, 3). They show the presence of 5-7 layers of tangentially elongated brownish cork cells (Ck) and occasional lenticels (L). Phelloderm (Pd) consists

Figure 1: ***Oxystelma esculentum*** (At column width)

Table 3: Phytochemical screening

Phyto constituent	Test	Result
Alkaloids	Dragendorff's test	-ve
	Wagner's test	-ve
	Mayer's test	-ve
	Hager's test	-ve
Flavonoids	Shinoda test	+ve
	Lead acetate test	+ve
Sterols	Salkowski test	+ve
	Libermann Buchardt test	+ve
Cardiac glycosides	Legal's test	+ve
	Baljet test	+ve
	Keller Killiani test	+ve
	Kedde's test	+ve
Saponin glycosides	Foam test	-ve
	Lead acetate test	-ve
Phenolics	Ferric chloride test	+ve
	Folin ciocalteu test	+ve
Sugars	Fehling's test	+ve
	Molisch test	+ve
Gums	Ruthenium red test	-ve

Table 4: Estimation of phytoconstituents

Phytoconstituent	% w/w± SD
Cardenolides	0.93 ±0.10
Phenolics	1.25±0.15
Flavonoids	0.45±0.13
Sugars	2.15±0.14

Number of observations = 5
SD = Standard Deviation

of stone cells, almost triangular or oval, isolated or in groups of 2-4, having U-shaped lumen. The cortex is narrow, consists of 4-6 layers of parenchymatous cells and stone cells (St) similar to those present in the phelloderm. (Figure 3A). 4-5 continuous bands of stone cells are present in the pericycle (Per) (Figure 3B). The stele of rhizome is large and consists of bicollateral vascular bundles having endarch xylem (X), whereas the vascular bundles of roots are collateral. The xylem vessels are mainly surrounded by thick-walled lignified xylem fibres. Xylem parenchyma cells are few (Figure 3D). Sheath of rosette crystals of calcium oxalate (CrS) is present above the xylem (Figure 3C). Parenchymatous pith (P) lies in the centre in rhizome, but is absent in root (Figure 3E).

Microscopy: Powder characteristics

It is a creamish white powder with no distinct odor or taste. The diagnostic feature of the powder is parenchymatous tissue filled with large number of rosette crystals of calcium oxalate. Stone cells, rectangular, oval or triangular in shape, isolated or in groups, sometimes with U-shaped lumen, were present in abundance. Xylem vessels with pitted, bordered pitted, reticulate and annular thickening were present. Cork in surface view was also visible (Figure 4).

CONCLUSION

A detailed study of roots and rhizomes of *Oxystelma esculentum* was performed. Microscopic study revealed the presence of diagnostic features like cork in surface view,

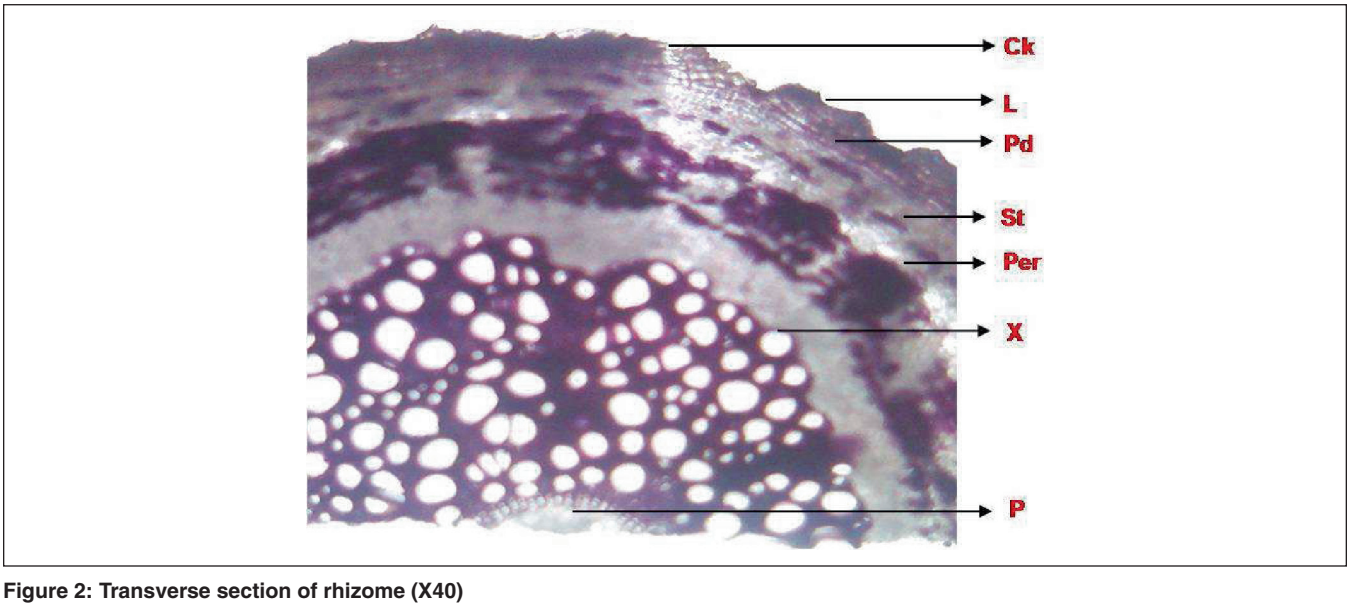

Figure 2: Transverse section of rhizome (X40)
Ck, Cork; L, Lenticel; Pd, Phelloderm, St, Stone cells; Per, Pericycle; X, Xylem; P, Pith. (At column width)

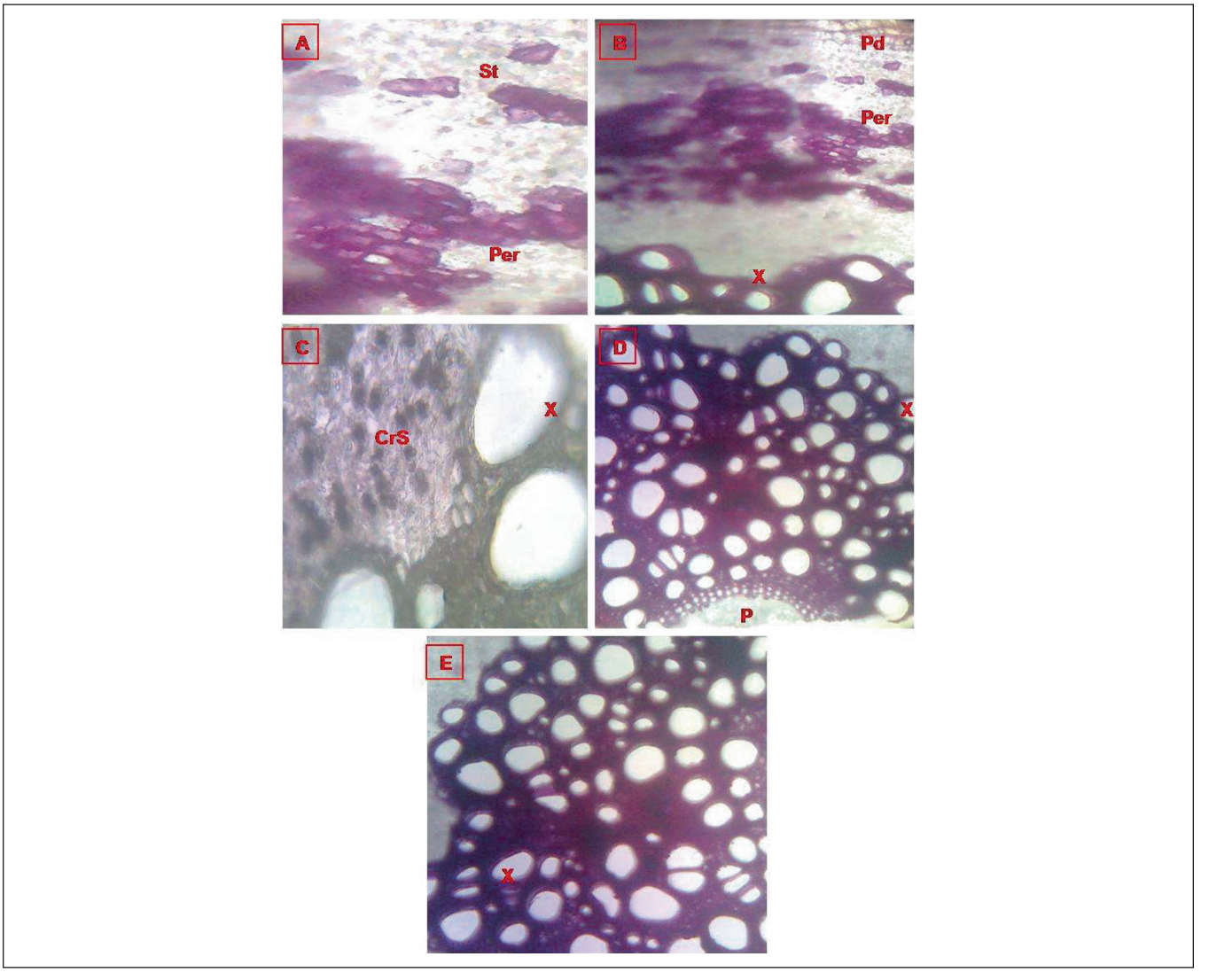

Figure 3: Enlarged portions of transverse section (X400)
A, Stone cells (St) in cortex and pericycle; B, Phelloderm (Pd), pericycle (Per) and xylem (X); C, Rosette crystal sheath (CrS) above xylem (X); D, Stele of rhizome having xylem (X) and pith (P); E, Root having xylem (X) but no pith.

stone cells, parenchymatous tissue having large number of calcium oxalate rosette crystals and xylem vessels with different types of thickening. Various quantitative microscopic parameters and physico-chemical parameters were established. Values of water soluble extractives were greater than those of alcohol soluble extractives, indicating the presence of water soluble phytoconstituents in higher amounts. Cardenolides, phenolics, flavonoids and sugars were detected by a thorough phytochemical screening procedure and they were estimated by their respective methods. This indicates that the plant can be useful for treating different diseases, especially those related to the cardiovascular system, as the therapeutic activity of a plant is always due to the presence of particular class of phytocompounds. The present study can serve as a useful gauge in the identification, authentication and standardization of the plant material as well as investigating its phytochemical composition, which would help in investigation of its possible pharmacological actions.

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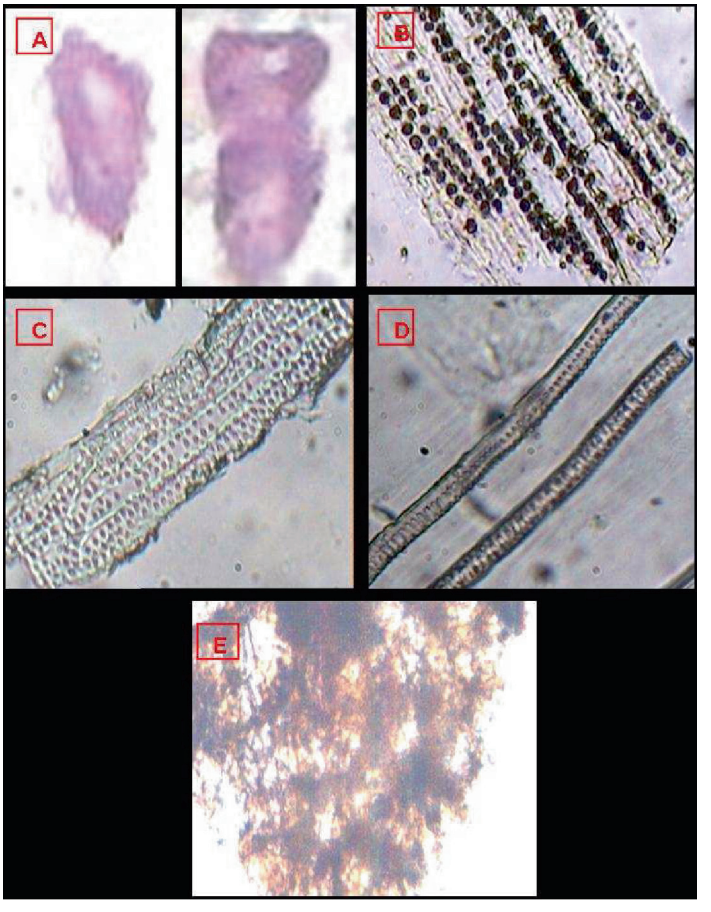

Figure 4: Powder study (X400)
A, Stone cells; B, Parenchymatous tissue filled with rosette crystals of calcium oxalate; C, Xylem vessels with pits and bordered pits; D, Xylem vessels with reticulate thickening; E, Cork in surface view.

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Herb – drug interaction of noni juice and *Ginkgo biloba* with phenytoin

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ABSTRACT

The common perception regarding the herbal medicines is that they are natural and safe. Although considered natural, most of the herbal medicine can interact with other drugs causing either potentially dangerous side effects or they can lead to loss or decreased therapeutic benefits of the drugs. Currently, there is growing concern to analyse and understand the herb – drug interactions. This study investigates effects of noni juice/*Ginkgo biloba* on the pharmacokinetics of phenytoin and also on the oxidative stress associated with long term administration of phenytoin. After pretreatment for 7 days with noni juice and *G.biloba*, on day 8 the phenytoin was co-administered orally with noni juice and *G. biloba* and the serum pharmacokinetics were determined at various time points (1, 2, 4,6, 8,12 and 24 h) by HPLC. The oxidative stress markers were determined after 30 days of treatment. Noni juice pretreated rats decreased the bioavailability of phenytoin by 2.81 fold, whereas *G. biloba* pretreated rats increased the bioavailability by 2.08 fold when compared with control. Noni juice and *G.biloba* treated rats provided significant protective effect against the oxidative stress induced by long term administration of phenytoin. It is observed that noni juice and *G.biloba* might have altered the bioavailability of phenytoin due to induction and inhibition of CYP2C9 enzymes.

Key words: Noni juice, *G.biloba*, Epilepsy, Herb – Drug interactions, Phenytoin; CYP2C9.

INTRODUCTION

Noni plant, *Morinda citrifolia* L. (Rubiaceae) is a small tropical evergreen shrub or tree indigenous to the Pacific Islands, South-east Asia, and various other tropical and temperate areas. All parts of the plant and especially fruits are used in herbal medicine by Polynesians for the past 2000 years.[1] According to traditional uses and based on recent scientific research, the noni plant has been proven to have broad range of therapeutic effects including antibacterial, antiviral, antifungal, anthelmenthic, analgesic, anti – hypertensive, anti – inflammatory, and immune enhancing effects.[2] Studies also reveal that it has anti-tubercular[3], anti-malarial activities.[4] The fruits are also known to have anti – HIV activity[5] and anticancer activity.[6] Noni is known to contain 3,3´ – bisdemethylpinoresicnol, americanol A, americanin A, americanoic acid A, morindolin, isopincepin, and damnacanthal (anthraquinone) as the active constituents.[7-8]

Ginkgo biloba L. (Ginkgoaceae) has been used for a very long time in traditional Chinese medicine. The standardized extract of *G.biloba* is referred to as EGb 761, which contains 24% flavonoids (including monosides, biosides, and triosides of quercetin, kaempferol, isorhamnetin and 3´-O-methylmyristicins), 6% terpenes (including the diterpenes like ginkgolide A, B, and C and the sesquiterpene bilabolide), organic acids (kynurenic acid, 6-hydroxykynurenic acid, vanillic acid, shikimic acid, glucuric acid) with trace amount of ginkgolic acids (0.0005%), minor constituents like proanthocyanidins, glucose and rhamnose.[9-14] Bridi *et al* 2001 reported that the antioxidant activity of *G. biloba* is due to flavonoid constituents and terpenoids are responsible for promotion of circulation.[15] *G. biloba* extract helps to protect neuronal membranes, facilitates better arterial circulation and improves electrical transmission in the brain.[16-18] *G. biloba* has beneficial effects on impaired brain glucose metabolism of streptozotocin – damaged rat brains, delays the progression of dependency and need for care in dementia patients.[19-21] *G. biloba* is reported to improve the decreased peripheral immune functions, schizophrenic symptoms, has stabilizing effect on the vestibular system

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of the ear[22-24] and there are also several reports on its anticancer activity.[25-32]

Deficiency of antioxidant status has been implicated in most of the neurological disorders and excessive lipid peroxidation is associated with teratogenicity and aggravation of seizures in epileptic patients.[33-36] Phenytoin and carbamazepine are the commonly prescribed antiepileptic drugs and studies depict that these antiepileptic drugs decrease antioxidant status in the body.[37-41] Studies have also revealed that phenytoin mono therapy leads to increased oxidative stress in the female epileptics.[42] In the pretext of avoiding the risks associated with oxidative stress, epileptics may tend to use the nutritional supplements rich in antioxidants.

The phenomenon of French paradox has drawn the attention of vast majority of people towards the beneficial effects of grape fruit juice and other functional foods. Similarly the use of fruit juices like orange juice, noni juice, star fruit juice and pomogranate juice etc., is increasing worldwide. Globally more than 200 companies are engaged in the commercial selling of noni juice and its products. Noni is currently distributed in more than 50 countries across the world, and its health benefits have been realized by millions of consumers.[43] Noni juice with its well balanced nutritional supplements containing vitamins, many trace minerals like calcium, magnesium and number of phytochemicals can act as a good antioxidant. It can enhance intercellular electrical signals in epileptic patients, which can prevent the seizure attacks and it can be the best choice to epileptics as an antioxidant. *G. biloba* extract is one of the most popular herbal medicines in the world which is known for improving cerebral circulation and cognitive functions.[44] Apart from its therapeutic use *Ginkgo* is also considered to be one of the important functional foods due to its rich flavonoid content. Noni juice and *G. biloba* can be used as alternative remedies for specific conditions like oxidative stress. These two herbal medicines i.e., noni juice and *G. biloba* were chosen for the present study in view of their rapid use as nutraceuticals and functional foods.

MATERIALS AND METHODS

Materials

The samples of Phenytoin and Carbamazepine were supplied as gift samples by Jubiliant Organosys (Noida, India). Diclofenac sodium and Chlorzoxazone were kind gift from Dr.Reddy's Laboratories (Hyderabad), Noni Juice (Cyber Noni) (Noni Connection Inc, USA) was purchased from the local dealer. G-KOBA (*Gingko biloba*) tablets were purchased from a local pharmacy, manufactured by Lessac Research Laboratories, Pondicherry, India. Each tablet of G-KOBA contained 40mg of dried extract of *G. biloba* (containing 9.6mg of ginkgoflavon glycosides). Ethyl acetate (Merck), Glacial acetic acid (Merck), Methanol (Merck) were purchased from the market. The study protocol was approved by the institutional animal ethical committee of Kakatiya University, Warangal, India. The HPLC was performed on LC – 10AT (Shimadzu Corporation, Kyoto Japan) system by injecting 20 µl of sample using Hamilton rheodyne syringe (Hamilton Bonaduz AG, Switzerland) into syringe loading sample injector (Model 7725i, Rheodyne LP, CA, USA.), diode array detector (Shimadzu SPD M10Avp model, Shimadzu Corporation, Kyoto, Japan), Biofuge Fresco centrifuge (Heraeus, Germany), cooling centrifuge (Remi Instruments, Mumbai, India), cyclomixer (Remi Instruments, Mumbai, India).

Experimental design

Noni juice/G. biloba – phenytoin interaction study

Male Wistar rats weighing 250 – 300 g were used for the study. Animals were maintained on regular rat feed of the animal house. The rats were divided into five groups of 6 animals each. The group 1 served as control. The group 2 was pretreated with 5ml/kg of noni juice for 7 days. The group 3 was pretreated with 100mg/kg of aqueous extract of *G.biloba* for 7 days. Group 4 was pretreated with 50mg/kg of fluconazole as CYP2C9 inhibitor for 7 days and the group 5 was pretreated with 50mg/kg of rifampicin as CYP2C9 inducer for 7 days. The rats were fasted for 16 hr prior to the study with water *ad libitum*. On day 8 the group 1 was administered orally phenytoin (20mg/kg suspended in 0.5% sodium carboxymethyl cellulose). Group 2 was administered orally with 5ml/kg of noni juice followed by phenytoin (20mg/kg suspended in 0.5% sodium carboxymethyl cellulose) after gap of 30 minutes. Group 3 was administered wi th 100mg/kg of aqueous extract of *G.biloba* followed by 20mg/kg of phenytoin after a gap of 30min. Group 4 was administered with 50mg/kg of fluconazole followed by 20mg/kg of phenytoin after a gap of 30 minutes. Group 5 was administered with 50mg/kg of rifampicin followed by 20mg/kg of phenytoin after a gap of 30 minutes. Blood samples were withdrawn by the retro - orbital vein puncture at different time intervals, viz. 0, 2, 4, 6, 8, 12 and 24 hours. The hypovolaemic condition was prevented by replacement with 0.5ml of normal saline after each sampling. Serum was separated and stored at – 20^0 C till the analysis.

Evaluation of the effect noni juice/G.biloba on CYP2C9 Substrate

Male Wistar rats weighing 250 – 300 g were used for the study. Animals were maintained on regular rat feed of the animal house. The rats were divided into three groups of

6 animals each. The group 1 served as control. The group 2 was pretreated with 5ml/kg of noni juice for 7 days. The group 3 was pretreated with 100mg/kg of aqueous extract of *G. biloba* for 7 days. The rats were fasted for 16 hours prior to the study with water *ad libitum*. On day 8 the group 1 was administered orally with 50mg/kg of diclofenac sodium (suspended 0.1% Tween 20); group 2 was administered orally with 5ml/kg noni juice followed by 50mg/kg of diclofenac sodium after a gap of 30min; group 3 was administered orally with 100mg/kg of *G. biloba* followed by 50mg/kg of diclofenac sodium after a gap of 30min. Blood samples were withdrawn by the retro - orbital vein puncture at different time intervals, viz. 0, 2, 4, 6, 8, 12 and 24 hours. The hypovolaemic condition was prevented by replacement with 0.5ml of normal saline after each sampling. Serum was separated and stored at – 20^0 C till the analysis.

HPLC analysis of Phenytoin

The serum concentrations of the phenytoin were determined by the HPLC assay method reported by Kishore *et al.,* 2003.[45] Stock solution of phenytoin (1mg/ml) was prepared in methanol, which was further diluted with methanol to the required concentrations viz., 0.5, 2.5, 10.0 and 50.0µg/ml of phenytoin. A standard graph was prepared by adding known concentration of phenytoin to drug free rat serum. Briefly, to each 100µl of serum sample, 100µl of each standard drug concentration and 20µl of internal standard (5µg/ml of carbamazepine dissolved in methanol) were added and extracted with 1.7ml of ethyl acetate, vortex mixed for 1 min and centrifuged at 3000 rpm for 8 min and supernatants were collected, evaporated to dryness, reconstituted with 100µl of mobile phase, vortex mixed for 1 min and 20µl was injected onto HPLC. The mobile phase consisted of a mixture of Methanol: Water: Acetic Acid (77:33:1 v/v/v). The mobile phase was degassed using ultrasonic bath (Model Sonorex, Bandelin Electronic, Germany). The analysis was performed isocratically at a flow rate of 1ml/min. The detector was operated at a wavelength of 230 nm and the data analysis was performed by LC Solution software (Shimadzu Corporation, Kyoto, Japan).

HPLC analysis of Diclofenac sodium

The serum concentrations of diclofenac sodium were determined by the modification of the HPLC method as reported by Raju *et al*., 2008.[46] Stock solution of diclofenac sodium (1mg/ml) was prepared in methanol. The stock solution was further diluted with methanol to the required concentrations viz., 0.1 – 50.0µg/ml. A standard graph was prepared by adding known concentration of diclofenac sodium to drug free rat serum. Briefly, to each 100µl of serum sample, 50µl of each standard drug concentration and 50µl of chlorzoxazone (internal standard 5µg/ml dissolved in methanol), 100µl of 2MHCl were added and vortex mixed for 3min. Then 3ml of chloroform was added and vortex mixed for 5min followed by centrifugation for 10 min at 3000rpm. The organic layer was separated, evaporated to dryness and reconstituted with 100µl of mobile phase and 20µl of the sample was injected onto HPLC. The mobile phase consisted of a mixture of Methanol: Ammonium Acetate Buffer (0.1M, pH4.2 adjusted with glacial acetic acid): Acetonitrile (60:30:10 v/v/v). The mobile phase was degassed using ultrasonic bath (Model Sonorex, Bandelin Electronic, Germany). The analysis was performed isocratically at a flow rate of 1ml/min and detector was operated at a wavelength of 280 nm. The data analysis was performed by LC Solution software (Shimadzu Corporation, Kyoto, Japan).

Pharmacokinetic analysis

Non compartmental pharmacokinetic analysis was carried out using the Kinetica TM software (Version 4.4.1, Thermo Electron Corporation, USA). The following pharmacokinetic parameters were calculated: C_{max}, observed maximum serum concentration for each serum sample; T_{max}, sampling time of the maximum serum concentration; $t_{1/2}$, terminal elimination half life; $AUC_{0 \text{ to } n}$, area under serum concentration/time plot until the last quantifiable value; AUC_{total}, area under serum concentration/time plot extrapolated to infinity, MRT, mean residence time of drug.

Determination of oxidative stress markers

The rats were divided into 5 groups of six animals each. All animals were treated for a period of 30 days. Animals in group 1 served as control (untreated), animals of group 2 were treated with 20mg/kg of phenytoin, animals in group 3 were treated with 5ml/kg of noni juice and animals in group 4 were treated with 5ml/kg of noni juice and 20mg/kg phenytoin. Animals of group 5 were animals were treated with 100mg/kg of *G. biloba,* animals in group 6 were treated with 100mg/kg of *G.biloba* and 20mg/kg of phenytoin. Blood samples were collected from the rats by retro orbital vein puncture at 5 day intervals; serum samples were separated and used for the determination of total antioxidant status and lipid peroxide levels.

Determination of total antioxidant status

The total antioxidant status in serum samples was determined by using DPPH method of Reddy *et al.,* 2004.[47] Ascorbic acid was used as a reference standard. The standard graph was prepared using different concentrations of ascorbic acid in water and the antioxidant status values were expressed in terms of nM of ascorbic acid.

Determination of lipid peroxides

The lipid peroxides in serum were measured by the method of Ohkawa *et al.,* 1979.[48] The standard graph for determination of malondialdehyde levels was prepared using 1,1,3,3 – tetraethoxy propane (TEP) reagent as the standard and the MDA content in the serum was expressed in nm/ml.

Statistical analysis

All the means are presented with their standard deviation (mean ± SD). All the parameters were compared between the control and pretreated groups using one way ANOVA, followed by post hoc Dunnet test.

RESULTS AND DISCUSSION

The serum concentration – time profile of phenytoin in different groups of rats is shown in Fig 1 and the pharmacokinetic parameters are presented in Table 1. The data shows that there was a significant decrease in the peak concentration (C_{max}) and bioavailability of phenytoin in noni juice pretreated group where as they were increased in case of *G. biloba* pretreated rats when compared with the control group. There was 3.0 and 3.20 fold decrease in C_{max} of phenytoin in group 2 and group 4, whereas there was 1.49 fold increase in group 3 and 1.44 fold decrease in group 5 respectively. The AUC_{total} and $AUC_{0\ to\ n}$ of phentytoin in noni juice pretreated group decreased to the extent of 2.81 and 4.02 fold respectively when compared to control. Where as the AUC_{total} and $AUC_{0\ to\ n}$ in *G.biloba* pretreated rats increased to the extent of 2.08 and 1.59 fold when compared to control. The AUC_{total} and $AUC_{0\ to\ n}$ of phentytoin in rifampicin pretreated group decreased by 5.25 and 7.04 fold when compared to control. The AUC_{total} and $AUC_{0\ to\ n}$ of phentytoin in fluconazole pretreated group increased by 1.09 and decreased by 1.26 fold when compared to control. The terminal half – life ($t_{1/2}$) in group 2, group 3, group 4 and group 5 was found to be decreased by 2.86, 2.63, 5.92 and 1.93 fold. The Mean Residence Time (MRT) in group 2, group 3, group 4 and group 5 was found to be decreased by 1.47, 1.19, 2.44, 1.10 fold. The t_{max} in the control and noni juice pretreated groups remained unaltered, while it was increased to 4h in *G.biloba* pretreated rats.

The serum concentration – time profile of diclofenac sodium is shown in Fig. 2 and the obtained pharmacokinetic parameters are presented in Table 2. The present study also demonstrates that there is a significant decrease in the peak concentration (C_{max}) and bioavailability of diclofenac sodium (a typical CYP2C9 substrate) in noni juice pretreated group, where as there was a significant increase in case of the *G.biloba* pretreated rats when compared with the control group. There was 1.81 fold decrease in C_{max} of diclofenac sodium in noni juice pretreated group, where as there was 1.77 fold increase in C_{max} of *G. biloba* pretreated rats. The AUC_{total} and $AUC_{0\ to\ n}$ of diclofenac sodium in noni juice pretreated group decreased to the extent of 2.92 and 2.71

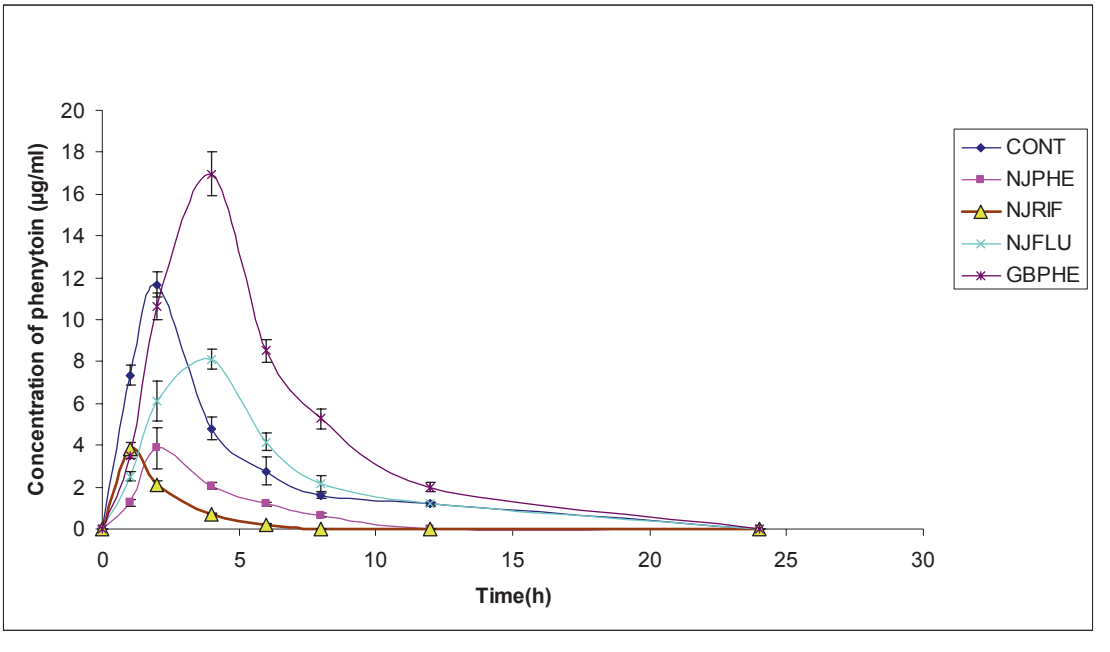

Figure 1: Mean serum concentration – time profile of phenytoin after oral administration of phenytoin to rats in different groups: PHE - control group, NJPHE - pretreated with noni juice, GBPHE - pretreated with *G.biloba*, RIFPHE - pretreated with rifampicin, FLPHE - fluconazole pretreated. Data are presented as mean ± standard deviation (SD) n = 6 for each group.

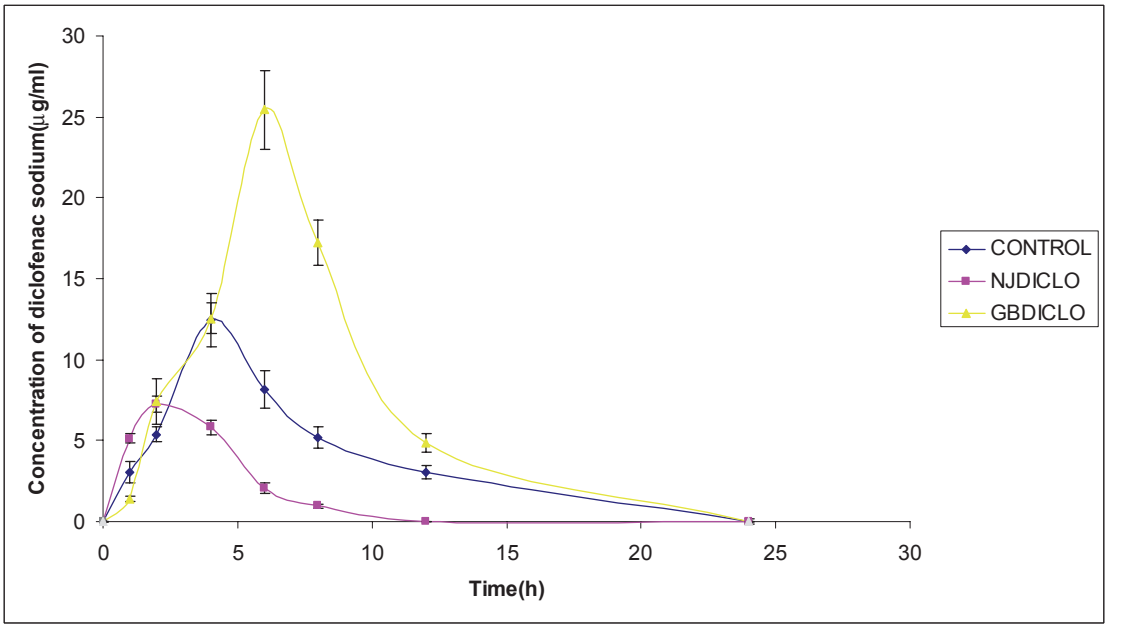

Figure 2: Mean serum concentration – time profile of diclofenac sodium after oral administration of carbamazepine to rats different groups: PHE - control group, NJPHE -pretreated with noni juice, GBPHE - pretreated with *G.biloba*, RIFPHE - pretreated with rifampicin, FLPHE - fluconazole pretreated. Data are presented as mean ± standard deviation (SD) n = 6 for each group.

Table 1: Pharmacokinetic parameters following oral administration of phenytoin in different groups of rats

Pharmacokinetic parameters	Control group	Noni juice pretreated group	Ginkgo biloba pretreated group	Rifampicin pretreated group	Fluconazole pretreated group
C_{max}(µg/ml)	11.69 ± 0.61	3.86 ± 0.98*	17.45 ± 1.51*	3.65 ± 0.49*	8.11 ± 0.49*
AUC_{total} (µg/ml/h)	45.76 ± 2.03	16.27 ± 1.76	96.07 ± 3.68*	8.28 ± 0.51*	50.24 ± 2.10
$AUC_{0\ to\ n}$ (µg/ml/h)	55.72 ± 4.19	13.84 ± 1.44*	88.70 ± 3.97*	7.91 ± 0.54*	44.21 ± 2.26
$t_{1/2}$(h)	7.11 ±1.52	2.48 ± 0.34*	2.70 ± 0.42*	1.20 ± 0.08*	3.67 ± 0.03
MRT (h)	7.03 ± 1.20	4.78 ± 0.38*	5.88 ± 0.41	2.38 ± 0.10*	6.38 ± 0.17

Values are Mean ± SD(n=6); *p < 0.05 compared to control

fold respectively. The AUC_{total} and $AUC_{0\ to\ n}$ of diclofenac sodium in *G. biloba* pretreated group increased to the extent of 1.96 and 1.71 fold respectively. The terminal half – life ($t_{1/2}$) in both groups was decreased to the extent of 3.80 and 1.92 fold respectively when compared to control. Similarly the Mean Residence Time (MRT) of diclofenac sodium in both the groups was decreased to the extent of 2.21 and 1.14 fold respectively when compared to control group. The t_{max} in the control, noni juice and *G.biloba* pretreated rats was found to 4, 2 and 6h respectively.

The total antioxidant status and lipid peroxide levels of the different groups of animals is shown in Fig. 3 and Fig. 4.

The present investigation brings to light that there is significant herb – drug interaction between noni juice/*G. biloba* with the commonly prescribed antiepileptic drug phenytoin, which is metabolized by CYP2C9 enzymes primarily to 5-(p-hydroxyphenyl-), 5-phenylhydantoin (HPPH).[49] The results show that noni juice decreased the bioavailability of phenytoin while *G. biloba* increased the

Table 2 : Pharmacokinetic parameters following oral administration of diclofenac sodium in different groups of rats (50mg/kg, orally)

Pharmacokinetic parameters	Control group	Noni juice pretreated group	*Ginkgo biloba* pretreated group
C_{max}(µg/ml)	12.44 ± 1.68	6.86 ± 0.29*	22.04 ± 2.43*
AUC_{total} (µg/ml/h)	93.17 ± 6.43	34.29 ± 1.42*	143.25 ± 6.82*
$AUC_{0\ to\ n}$ (µg/ml/h)	73.05 ± 4.09	31.84 ± 2.24*	159.76 ± 7.17*
$t_{1/2}$(h)	4.56 ±0.93	1.73 ± 0.39*	2.38 ± 0.23*
MRT (h)	8.52 ± 1.16	3.84 ± 0.24*	7.44 ± 0.26

Values are Mean ± SD (n= 6); * p<0.05 compared to control

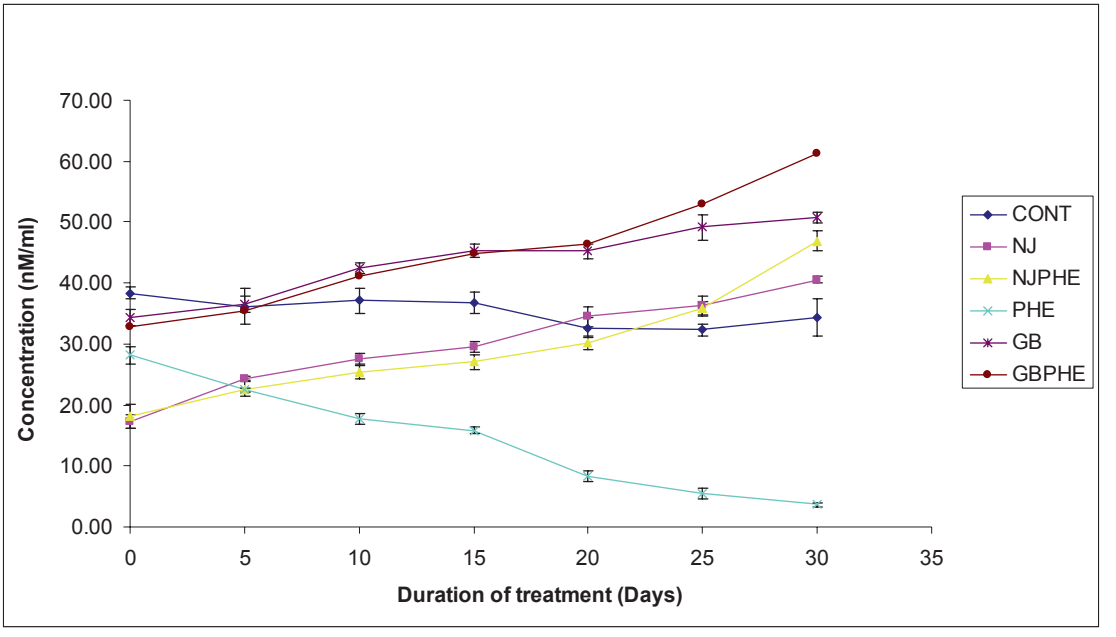

Figure 3: Total antioxidant status in rats of four different groups: All groups are treated for 30 days. CONT: control group (untreated); PHE: treated with phenytoin; NJ: treated with noni juice; NJPHE: treated with noni jucie and phenytoin; GB: treated with *G.biloba*; GBPHE: treated with *G.biloba* and phenytoin. Total antioxidant status was measured in nM/ml of Ascorbic acid. . Data are presented as mean ± standard deviation (SD) n = 6 for each group.

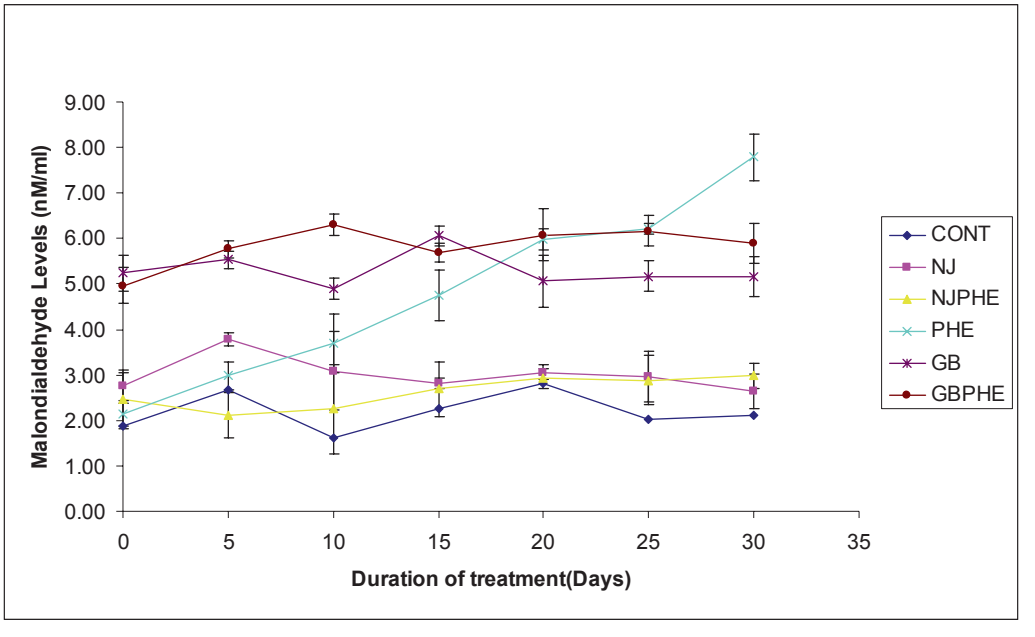

Figure 4: Serum lipid peroxides (MDA) levels in different groups of rats: All groups are treated for 30 days. CONT: control group (untreated); PHE: treated with phenytoin; NJ: treated with noni juice; NJPHE: treated with noni jucie and phenytoin; GB: treated with *G.biloba*; GBPHE: treated with *G.biloba* and phenytoin. Lipid peroxidation was measured in nM/ml of MDA. . Data are presented as mean ± standard deviation (SD) n = 6 for each group

bioavailability of phenytoin. Since there is significant difference in $t_{1/2}$ of noni juice/*G.biloba* pretreated rats when compared to control the possibility of involvement of intestinal absorption, intestinal metabolism and hepatic metabolism exists. The involvement of P-glycoprotein (P – gp) in noni juice pretreated rats may be ruled out because phenytoin is a weak inhibitor of p-gp.[50] If the involvement of P-gp is speculated then the bioavailability of phenytoin should have been increased but in the present study the bioavailability of phenytoin is decreased. Hence

the involvement of intestinal and hepatic CYP2C9 induction might be the possible mechanism for the decrease in the bioavailability of phenytoin. Further, the results also show that the % reduction in bioavailability of phenytoin in rats pretreated with noni juice is almost similar to the % reduction in bioavailability of rats pretreated with CYP2C9 inducer (rifampicin), which indicates that noni juice has similar type of inductive effect on CYP2C9 enzyme. Till date, there are no reports on the interaction potential of noni juice with drugs except that it increases the coumadin resistance due to the presence high amount of potassium ions in it. The actual constituents responsible for the observed interaction cannot be specified because the noni juice contains several phytoconstituents. The exact mechanism by which noni juice modulates phenytoin metabolism is currently unknown because the fruit juices, alcoholic beverages, teas, and herbal extracts are complex chemical mixtures and it is very difficult to determine which compound or compounds are responsible for the potential interaction with metabolizing enzymes leading to herb – drug interactions.[51] The phenolic functional micronutrients of noni juice such as damnacanthal, scopoletin, morindone, alizarin, aucubin, nordamnacanthal, rubiadin, rubiadin-1-methyl ether and other anthraquinone glycosides might have led to the present interaction. As most of the anthraquinone derivatives have laxative effect, it may be speculated that anthraquinones present in noni juice might have lead to increased elimination rate of phenytoin. There is also a possibility that the anthraquinone derivatives and other active constituents of noni juice may lead to induction of hepatic microsomal enzymes. In case of *G.biloba* pretreated rats the % increase in the bioavailability of phenytoin is similar to the % increase in bioavailability of rats pretreated with fluconazole (CYP2C9 inhibitor) and the increase in bioavailability of phenytoin can also be attributed to inhibition of P-glycoprotein (P-gp) because phenytoin is known to be a weak inhibitor of P-gp. *G. biloba* is reported to alter the pharmacokinetics of theophylline by induction of CYP1A2, propranolol by induction of CYP2B1/2 and CYP3A1.[52-53] *G. biloba* extract is proven to reduce the therapeutic potency of phenobarbital due to induction of CYP2B enzymes.[54] Shinozuka *et al.,* 2002[55] reported that feeding of *G. biloba* extract for 4 weeks significantly reduced the hypotensive effect of nicardipine which is metabolized by CYP3A4 in rats. Yoshioka *et al.,* 2004[56] reported that *G.biloba* extract significantly inhibits the metabolism of nifedipine which is a typical CYP3A4 substrate. Sugiyama *et al.,* 2006[57] reported that pretreatment with *G. biloba* extract significantly reduced the hypoglycemic action of tolbutamide in elderly rats due to induction CYP2C9. Umegaki *et al.,* 2002[58] reported that pretreatment of rats with *G. biloba* increased the concentration and activity of various CYP enzymes (containing CYP1A1, CYP1A2, CYP2B, CYP2E1 CYP3A and CYP2C9) in the rat liver. The studies of Gaudineau *et al.,* 2004[59]; He and Edeki 2004[60] and Numa *et al.,* 2007[61] proved that the active constituents of *G. biloba* have CYP2C9 inhibitory potential and the present results are in agreement with these reports. In contrast, our results are in contradictory to the results of Sugigyama *et al.,* 2004 and Umegaki *et al.,* 2002. The actual constituents responsible for the increased bioavailability of phenytoin in *G. biloba* pretreated rats cannot be specified because it contains 30 different flavonoid compounds.[62] In both the noni juice and *G. biloba* pretreated rats the same type inhibitory and inductive effect was observed on the pharmacokinetics of diclofenac sodium (a typical CYP2C9 substrate). Hence it may be speculated that noni jucie has inductive effect where as *G. biloba* has inhibitory effect on CYP2C9. The total antioxidant status in the rats pretreated with phenytoin alone decreased significantly when compared rats co - administered with noni juice and phenytoin/aqueous extract of *G. biloba* and phenytoin. The lipid peroxide levels were found to be more in the rats pretreated with phenytoin alone when compared rats co - administered with noni juice and phenytoin/aqueous extract of *G. biloba* and phenytoin. These results clearly show that the polyphenolic constituents of noni juice and the flavonoids present in the *G. biloba* aqueous extract offer significant protection against the oxidative stress induced by long term administration of phenytoin.

In conclusion it is observed that there is significant herb – drug interaction between noni juice/*G.biloba* with phenytoin. However, additional studies are required to define which active constituents in noni juice/*G. biloba* are responsible for induction and inhibition of CYP isozymes.

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Anti-inflammatory and Analgesic Properties of Ethanolic Stem Bark Extract of *Ficus trichopoda* in Rats.

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ABSTRACT

Introduction: *Ficus trichopoda* is a ficus species growing in wet places – swamp forest, river banks and swamp grassland. It is well used in the management of inflammation - related conditions locally. This present work was undertaken to investigate anti-inflammatory and analgesic properties of aqueous ethanolic extract of *Ficus trichopoda* bark in rats. **Methods:** The anti-inflammatory effect was investigated by using the acute inflammatory model of carrageenan - induced paw oedema and its analgesic activity using formalin test and tail flick test in rats. **Results:** The study for preliminary phytochemical secondary metabolites revealed the presence of saponins, tannins, alkaloids and free amines/amino acids. Oral administration of the aqueous ethanolic extract of *F. trichopoda* in doses up to 5000 mg/kg body weight did not produce any mortality and any visible signs of toxicity. *Ficus trichopoda* extract at the doses (125 - 500 mg/kg) showed significant ($p < 0.05$) dose dependent inhibition of oedema formation in the carregeenan induced model. The extract showed significant dose dependent inhibition of the inflammatory (late) phase but not the neurogenic (early) phase of the formalin test in rats. The analgesic activity in tail flick method showed significant ($p < 0.05$) elevation in pain latency threshold from 30, 60, 90 and 120 minutes after pretreatment. **Conclusion:** The results obtained suggest marked analgesic and anti-inflammatory activity of ethanolic extract (125- 500 mg/kg). This finding supports that the stem bark is useful in inflammatory and painful conditions.

Key words: Bark, *Ficus trichopoda*, Inflammation, Pain, Phytochemicals

INTRODUCTION

Medicinal plants have long been used in traditional medicine for therapeutic purposes and their healing effects have well been recognized since ancient times. *Ficus trichopoda* Baker is a medicinal plant belonging to the Moraceae family (commonly called fig trees) used popularly as a 'multi-purpose' medicinal plant in Uganda. Forty-four species are known from Uganda.[1-2]

Ficus trichopoda is a ficus species growing in wet places – swamp forest, river banks and swamp grassland. The vernacular name is 'kaboga' in Luganda language of Uganda. It is widely used for live fence, building poles, fibre, firewood and ground water.[3] The majority of the medicinal uses of figs in humans are based on historical reports or anecdotal evidence with only a few reports coming from modern clinical trials. The reported medicinal properties of different *Ficus* species includes: anti-inflammatory, antineoplastic, antioxidant, antiulcer and antidiarrheal.[4] Species of the genus *Ficus* (Moraceae) are used in many parts of East Africa for the treatment and management of many clinical and gynaecological problems.[5-6]

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Despite the popular use of these species in management of inflammation – related conditions locally, there are no literatures on biological studies addressing the anti-inflammatory and analgesic effects of *F. trichopoda*. This study reported the investigation into the anti-inflammatory effects of the plant extract using carrageenan-induced oedema in rats, and its analgesic activities using formalin (chemical) and thermal (tail immersion) pain induction methods.

MATERIALS AND METHODS

Plant collection and extract preparation

The bark of *F. trichopoda* was collected in November 2009 in Ishaka, Western Uganda. The plant was identified by its local name and was later authenticated at the Botany Department of Makerere University Kampala. A voucher specimen was prepared and deposited at the Kampala International University, School of Pharmacy herbarium.

The plant bark was air dried at room temperature and later ground to powder. The air- dried powdered bark of *F. trichopoda* was extracted with 70% ethanol by cold maceration for 48 hours with occasional shaking. The extract was concentrated by evaporation and dried in an oven at 40^0C to obtain a brown solid powder. A freshly prepared solution in distilled water was used for pharmacological studies.

Animals

Male Wistar rats weighing 120 to 200g and male Swiss albino mice weighing 25 to 40g were obtained from the Pharmacology department laboratory animal facility. Animals were maintained in ordinary animal cages under constant 12 h/12 h light/dark cycle. They were acclimatized in the laboratory environment for at least two weeks, and were fed with standard pellet diet (Nuvita animal feed Ltd, Uganda) and water *ad libitum*. All experiments were carried out with strict compliance to The "Principle of Laboratory Animal Care" (NIH Publication No. 85-23)[7] and ethical guidelines for investigation of experimental pain in conscious animals.[8]

Drugs and Reagents

Indomethacin (MSD, Canada), Carrageenan (Sigma Chemical Co., USA) and Formaldehyde (M & B, UK) were used.

Phytochemical Screening

Conventional standard protocols (Trease and Evans, 1983)[9] for detecting the presence of different chemical constituents in the plant extract were employed. Secondary metabolites tested include alkaloids, tannins, saponins, glycosides, flavonoids, digitalis, and phenols.

Acute toxicity study

Acute toxicity study was carried out using the method of Lorke[10] with slight modification using the oral route. In the first phase, nine mice randomly divided into three groups of three mice per group were given 500, 1000 and 5000 mg/kg body weight of ethanol extract of *F trichopoda* orally (via a cannula), respectively. The mice were observed for signs of adverse effects and death for 24 h and then weighed daily for 14 days. The geometric mean of the least dose that killed mice and the highest dose that did not kill mice was taken as the median lethal dose.

Carrageenan-induced paw oedema in rats

Pedal inflammation in rats was produced according to the method described by Winter et al.[11]. Animals in treatment groups were orally administered with 125, 250, and 500mg/kg of ethanol extract of *F. trichopoda*, and indomethacin (10 mg/kg). At the same time, control animals received 10 ml/kg saline. One hour later, all animals were injected with 0.1ml of 1% carrageenan in the right hind foot under the subplantar aponeurosis. Measurement of paw size was carried out by gently wrapping a piece of white cotton thread round the paw, and measuring the circumference on a metre rule. This method has been successfully used in previous studies.[12, 13, 14] Measurements of paw (oedema) sizes were carried out just before, and at hourly intervals for five hours after carrageenan injection. Readings were taken at least twice on each occasion and the mean value for each reading was recorded.

Many studies showed that oedema in the right hind paw of rats induced with carrageenan peaks at third hour following the inhibitory activity of test agents and indomethacin against carrageenan –induced rat paw oedema. The inhibitory activity was calculated using the following formula:

$$\text{Percentage inhibition} = \frac{(Ct - Co)\ \text{control} - (Ct - Co)\ \text{treated}}{(Ct - Co)\ \text{control}} \times 100$$

where Ct – paw (oedema) sizes at time 3h after carrageenan injection and Co – paw (oedema) sizes before carrageenan injection.

Formalin test

This test was carried out in five groups of rats (n = 5) pretreated with either saline; the extracts (125, 250 and 500 mg/kg p.o.) and indomethacin. Formaldehyde (0.05 ml,

2.5%) was injected into the sub-plantar surface of the rat left hind paw 30 min after treatment. Severity of pain was rated using Dubuisson and Dennis,[15] pain scoring measurements in the following manner: (0), normal weight bearing on the injected paw; (1), light resting of the paw on the floor; (2), elevation of the injected paw; and (3) for licking, biting and grooming of paw. These responses were observed and recorded for a total of 60 min. The first 10 min were considered as the early phase and represents aphasic pain while the period between 15 and 60 min was recorded as the late phase (representing tonic pain).

Tail-Flick test

The study as described by Yaro et al[16] was adopted with minor modification for our local laboratory settings. The rats were initially screened for the test by immersing about 2cm of their tails into hot water maintained at 50 ± 1°C. The animals that lifted their tail within 5 seconds were selected for the study.

Twenty five rats that shows response (withdrawing the tail within 5 s) were selected and grouped into five (n = 5). The rats were treated with extracts (125, 250 and 500 mg/kg), indomethacin (10 mg/kg), or saline (10 ml/kg). The tail withdrawal reflex period (latency/pain threshold) was taken at 30, 60, 90 and 120 minutes after the administration for each rat.

Data analysis

Results were expressed as mean ± standard error of mean (SEM). Student t-test was used to analyze level of statistical significance between groups and Analysis of Variance (ANOVA) among groups. All level of significance were set at $p < 0.05$.

RESULTS

Phytochemical Screening

Phytochemical analysis of the crude extract gave positive reactions for the following secondary metabolites: saponins, tannins and alkaloids, free amines/amino acids. Glycosides, flavonoids, digitalis, phenols, resins and volatile oils were absent.

Acute toxicity studies

Oral administration of the aqueous ethanolic extract of *F. trichopoda* in doses up to 5000 mg/kg body weight did not produce any mortality and any visible signs of toxicity when observed up to 72 hrs after administration. It was observed that there was no weight loss, no loss of appetite, and no mortality up to 14 days after treatment.

Carrageenan-induced paw oedema in rats

The result obtained from this experiment is shown in Table 1. Pre-treatment with Extract (125 – 500 mg/kg) and indomethacin (10 mg/kg) produced statistically significant ($p<0.05$) dose dependent inhibition of the oedematous response. At the third hour post-carrageenan, oedema was inhibited by 19.23, 23.08, and 38.46% by 125, 250 and 500 mg/kg of extract respectively. Indomethacin administered at 10mg/kg inhibited oedema by 61.53%.

Formalin test

The extract exhibited a dose dependent howbeit insignificant inhibition of the early phase of the formalin test showing a pain inhibition of 26.96, 37.39 and 46.09%. In the late phase (15 – 60 mins), pain inhibition was significant ($P < 0.05$) showing % inhibition of 41.13, 61.47, and 60.60%

Table 1: Effect of ethanolic extract of *Ficus trichopoda* bark (FTB) on carrageenan-induced paw oedema in rats.

Treatment	Dose (mg/kg)	Paw Sizes (cm) at					
		0 hour	1 hour	2 hour	3 hour	4 hour	5 hour
Control Distilled water	10 ml/kg	2.22 ± 0.04	2.60 ± 0.03	2.60 ± 0.03	2.74 ± 0.02	2.72 ± 0.06	2.70 ± 0.04
FTB Extract	125	2.24 ± 0.05	2.56 ± 0.02	2.64 ± 0.04	2.66 ± 0.02* (19.23)a	2.66 ± 0.05	2.70 ± 0.07
FTB Extract	250	2.22 ± 0.07	2.54 ± 0.05	2.66 ± 0.06	2.62 ± 0.02* (23.08)a	2.50 ± 0.06	2.54 ± 0.04
FTB Extract	500	2.26 ± 0.04	2.42 ± 0.06	2.56 ± 0.05	2.58 ± 0.04* (38.46)a	2.64 ± 0.06	2.50 ± 0.05
Indomethacin	10	2.24 ± 0.02	2.52 ± 0.04	2.48 ± 0.09	2.44 ± 0.05* (61.53)a	2.46 ± 0.05	2.44 ± 0.05

Each value is the mean ± SEM of five rats (n = 5)
*Each value is significant at $p < 0.05$, compared with control using the Student's t – test.
[a]Inhibition (%) of oedema in treated group vs control

for 125, 250 and 500 mg/kg of the extract respectively (Table 2). Higher pain inhibition was, however, observed in the late phase.

Tail immersion test

The results of this test are shown in Table 3. There was significant (P< 0.05) dose dependent increase in pain response threshold against heat induced stimulus. This effect started 30 mins after treatment and persisted throughout the 120 min duration of the experiment.

DISCUSSION

The absence of death in the groups of mice treated with *Ficus trichopoda* bark extract at doses up to 5000 mg/kg body weight is suggestive that the extract is practically non –toxic acutely and is relatively safe with low risk of acute intoxication. This study evaluated the scientific basis for the traditional use of *Ficus trichopoda* against inflammation and pain. The anti-inflammatory and analgesic effects were analysed using different stimuli such as chemical agents (carrageenan, formalin) and heat (tail-flick). The results demonstrated a modest but significant anti-inflammtory activity of the ethanolic extract of *Ficus trichopoda* bark (19.23, 23.08, and 38.46 %) as compared to indomethacin (61.53 %). The carrageenan-induced rat paw oedema model is a significant predictive test for anti-inflammatory agents acting by the mediators of acute inflammation, the result of this study is an indication that *F. trichopoda* can be effective in acute inflammatory disorders. Formalin as a potent oedematous agent produced inflammation through the release of several inflammatory mediators including prostaglandins.[17] Sub-cutaneous injection of formalin produces distinct biphasic pain, termed early and late phases. Drugs that act primarily on the central nervous system inhibit both phases equally while peripherally acting drugs inhibit the late phase.[18] The formalin test for ethanolic extract of *Ficus trichopoda* bark demonstrated a significantly (P < 0.005) higher percentage of inhibition in the late phase (inflammatory phase). The tail immersion test is a thermal painful stimuli model and is selective for the evaluation of centrally, but not peripherally acting analgesic drugs.[19] Our findings showed significant dose-dependent increase in latency period of response to heat. The data concerning the analgesic effects of the ethanolic extract of *Ficus trichopoda* bark is indicative of morphine -like[20] and also NSAIDs-like effects by inhibiting cyclooxygenase in peripheral tissues thereby interfering with the mechanism of transduction in primary afferent nociceptors.[21]

The ability of the extract to reduce the size of oedema produced by carrageenan and the paw licking in formalin test, suggests that it contained chemical component(s) that may be active against inflammatory conditions.

Table 2: Effect of the ethanolic extract of Ficus trichopoda bark (FTB) on formalin –induced nociception in rats.

Treatment	Dose (mg/kg)	Score of pain[a]			
		0 -10 mins	% inhibition	15- 60 mins	% inhibition
Control Distilled water	10 ml/kg	2.30 ± 0.31	-	2.31 ± 0.18	-
FTB Extract	125	1.68 ± 0.19	26.96	1.36 ± 0.09*	41.13
FTB Extract	250	1.44 ± 0.27	37.39	0.89 ± 0.12*	61.47
FTB Extract	500	1.24 ± 0.20	46.09	0.91 ± 0.94*	60.60
Indomethacin	10	1.44 ± 0.29	37.39	1.06 ± 0.12*	54.11

[a]Each value is the mean ± SEM of five rats (n = 5)
*Each value is significant at p < 0.05, compared with control using the Student's t – test.

Table 3: Effect of ethanolic extract of Ficus trichopoda bark (FTB) on tail immersion test in rats

Treatment	Dose (mg/kg)	Mean Reaction Latency (s)[a]			
		30 mins	60 mins	90 mins	120 mins
Control	Saline	4.64 ± 0.14	4.01 ± 0.19	3.62 ± 0.17	3.70 ± 0.20
FTB Extract	125	4.85 ± 0.15	5.78 ± 0.10*	5.47 ± 0.12*	5.21 ± 0.10*
FTB Extract	250	6.43 ± 0.17*	6.65 ± 0.10*	6.53 ± 0.08*	6.81 ± 0.06*
FTB Extract	500	6.70 ± 0.08*	6.97 ± 0.14*	6.95 ± 0.07*	6.87 ± 0.12*
Indomethacin	10	6.91 ± 0.13*	7.29 ± 0.07*	7.24 ± 0.11*	7.23 ± 0.07*

[a]Each value is the mean ± SEM of five rats (n = 5)
*Each value is significant at p < 0.05, compared with control using the ANOVA.

The demonstrated analgesic and anti-inflammatory activities may be due to the presence of saponins, tannins and alkaloids either singly or in combination in the ethanolic extract of *F trichopoda* bark as demonstrated also by Zaku et al.,[22] in the bark extract of *F. racemosa*.

In conclusion, the ethanolic extract of *Ficus trichopoda* bark presented with an anti-inflammatory and analgesic effects and this supports the use of this plant in folk medicine in treatment of inflammatory pain.

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Anti-inflammatory and Analgesic activities of root and stem of *cissus rependa* vahl

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ABSTRACT

Anti-inflammatory and analgesic activity of root and stem of *Cissus rependa* Vahl.(Vitaceae), a folklore medicinal plant of Gandhamardana hill ranges of Orissa, was carried out on albino rats and mice at the dose of 180 mg/kg and 260 mg/kg respectively. The effect on inflammation was studied on carrageenan and formalin induced paw oedema models and compared with phenylbutazone and diclofenac sodium standards respectively. Analgesic activity was evaluated in formalin induced paw licking and tail flick method where indomethacin and pentazocine were used as standard drugs respectively. The *C. rependa* root (CRR) significantly inhibited carrageenan induced paw oedema, formalin induced paw licking and prolonged tail flick response, however it was failed to suppress the formalin induced paw oedema, whereas *C. rependa* stem (CRS) significantly inhibited carrageenan induced paw oedema and formalin induced paw oedema at 24h, however it failed to inhibit formalin induced pain response. Further, CRS prolonged tail flick response significantly only at 180minutes. The results suggest that the root of *C rependa* has significant analgesic and anti-inflammatory potential as reflected by the parameters investigated, while stem has weak anti-inflammatory and analgesic activity. Thus it can be concluded that roots of *C rependa* have significant anti-inflammatory and analgesic activity and can be preferred in the treatment of pain and inflammation.

Keywords: *Cissus rependa*, folklore, Gandhamardan hill, analgesic, anti-inflammatory.

INTRODUCTION

Plants are being used in the traditional systems of medicine in many parts of the world, especially in rural communities, for the control, management and treatment of a variety of human and animal ailments. The use of leaves, stems, barks, flowers, seeds or roots is common among people, especially by the tribal people to manage various ailments. *Cissus repanda* Vahl.(Vitaceae) is a medicinal plant distributed from Kuman to Arunachal Pradesh, Tripura, Assam, Bihar, Orissa, Madhya Pradesh, and Western Ghats region up to 1350 meters[1]. The root and stem of this plant has been used among the tribal peoples of Gandhamardana hill ranges, Bolangir, Orissa for its properties of alleviation pain from cutting wound, and bone fractures.[2, 3.4.]. In spite of its reputation in treating these ailments, till date no pharmacological work to support these claims has been reported. Hence the present study was under taken to evaluate the anti-inflammatory and analgesic activities of root and stem of *Cissus rependa* Vahl. in albino rats and mice.

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MATERIALS AND METHODS

Plant material

Fresh plant material was collected from its natural habitat of Gandhamardana hill ranges, Bolangir, Orissa, India. The plant was authenticated by pharmacognosist. The verified specimen was preserved in the departmental herbarium museum (vide no. 6001/2009) for future references. The root and stem were cutout separately and washed thoroughly. The cleaned material was shade dried and powdered (Mesh 80). The *C. rependa* root was coded as **CRR** and *C. rependa* stem as **CRS** and stored in air tight container for experimental study.

Experimental animals

Wistar strain albino rats of either sex weighing 200 ± 20g and Swiss albino mice of either sex weighing 30 ± 06g were procured from the animal house attached to our institute (Registration No.548/2002/CPCSEA). They were housed in polypropylene cages and fed with Amrut brand rat pellet feed supplied by Pranav Agro Industries and tap water given *ad libitum*. The animals were acclimatized for at least one week in lab conditions before the commencement of experiment in standard laboratory conditions 12 ± 01 hour day and night rhythm, maintained at 25 ± 3°C and 40 to 60 % humidity. Before the test, the animals were fasted for at least 12 hours. Institutional animal ethics committee had approved the experimental protocol (Approval number; IAEC 03/08-10/Ph.D-06) and the care of animals was taken as per the CPCSEA guidelines.

Dose selection

The dose fixation for the experimental animals was done on the basis of body surface area ratio by referring to the standard table of Paget and Barnes (1964)[5]. The adult human dose (2g per day) was converted to animal dose. On this basis the rat dose was found to be 180 mg/kg and for mouse 260 mg/kg. The test drug was suspended in distilled water with suitable concentration depending up on body weight of animals and administered orally with the help of gastric catheter sleeved to syringe. The drugs were administered to overnight fasted animals.

ANTI-INFLAMMATORY ACTIVITY

Carrageenan induced rat paw oedema

The Wister strain albino rats of either sex were weighed and randomly divided in to four groups of six each. First group received distilled water and served as control group. The second and third groups received test drugs CRR and CRS respectively. Fourth group was administered with standard anti-inflammatory drug phenylbutazone (Wilson Laboratories, Mumbai) in the dose of 100mg/kg. The vehicle and test drugs were administered to the respective groups for five consecutive days; whereas standard drug was given only once i.e., one hour before the carrageenan injection[6].

Initially left hind paw volumes up to the tibio-tarsal articulation were recorded prior to Carrageenan injection by using plethysmograph[7]. The plethysmograph employed, consisted of 10 ml glass vessel (25mm × 65mm) fixed to 2 ml glass syringe through pressure tubing. About 4 ml of Mercury was filled in the syringe and the mercury level was adjusted to zero mark on the micropipette. The space between the zero mark and the fixed mark on the glass vessel was filled with water and few drops of teepol. The initial level of fluid was adjusted and set at zero. The paw was immersed in water exactly up to the tibio-tarsal articulation. The increased level of water in the glass vessel was adjusted to the prefixed mark by releasing the pressure of the connected syringe. The level where water and mercury interface in the micropipette was recorded as paw volume.

On fifth day one hour after drug administration oedema was produced by injecting 0.1 ml freshly prepared 1% carrageenan in sterile saline solution to the sub-plantar aponeurosis of the left hind limb. The rats were administered tap water in the dose of 2 ml per 100 g body weight to ensure uniform hydration and hence to minimize variations in oedema formation. Paw volume was recorded three hour after carrageenan injection. Results were expressed as an increase in paw volume in comparison to the initial paw volumes and also in comparison with control group.

Formaldehyde induced paw oedema

The test conditions and groupings were similar to carrageenan induced paw oedema as mentioned above, except the standard anti-inflammatory drug used (diclofenac sodium - 5mg/kg - Novartis India Limited). Pedal inflammation was induced by injecting 0.1 ml of 3 % formaldehyde solution below the plantar aponeurosis of the right hind paw of the rats. The paw volume was recorded immediately prior to compound administration (0 h) and then at 24 and 48 h after formaldehyde injection[8]. Results were expressed as an increase in paw volume in comparison to the initial paw volumes and also in comparison with control group.

ANALGESIC ACTIVITY

Tail flick test

Mice were placed on the tail flick unit so that constant heat intensity was applied to the lower third of the animal's tail. When the animal flicked its tail in response to the noxious stimulus both the heat source and timer were stopped. A cut off time of 10 seconds was set to avoid tail damage. Thus basal reaction time of each mouse to radiant heat was recorded and those having TFL (tail flick latency) less than 10 seconds were selected. Thus selected mice were randomly divided in to four groups of six each. First group received similar volume of vehicle as test drug and served as normal control. Mice in group two and three were treated with CRR and CRS respectively. To the group four standard analgesic drug pentazocine (20mg/kg i.p. - Ranbaxy laboratories) was administered. The vehicles test drug and

reference standard were administered to the respective groups one hour prior to experiment. The TFL was recorded at 30, 60, 90, 120, 180 and 240 minutes[9].

Formalin induced hind paw licking

Animal grouping and test drug administration are similar to carrageenan induced paw oedema model. Indomethacin (10mg/kg orally - Cipla) was used as standard analgesic drug. Pain was induced by injecting 0.1 ml of 3% formalin in distilled water in subplantar region of right hind paw and the duration of paw licking as an index of nociception was counted in periods of 0 to 10 minutes (Early phase) and 20 to 30 minutes (Late phase)[10].

Statistical analysis

Data are expressed as mean ± SEM. Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Dunnett's multiple 't' test) and also by unpaired Student 't' test. The level of significance was set at $P<0.05$ and the level of significance was noted and interpreted accordingly.

RESULTS

Both CRR and CRS at the dose of 180mg/kg, significantly inhibited paw oedema at both 3 and 6 hours after carrageenan injection. The observed inhibition is almost equal to standard anti-inflammatory drug Phenylbutazone (Table-1).

The result (Table - 2) shows that CRR at the dose of 180 mg/kg did not suppress the formalin induced paw oedema at both 24 and 48 hour intervals. CRS at the dose of 180 mg/kg has significant reduction in the formalin induced paw oedema at 24 hour, where as it did not suppress the paw oedema at 48 hour intervals. Diclofenac sodium suppressed the formalin induced paw oedema in significant manner at both 24 and 48hours comparison to control group.

Table - 3 shows the results of the formalin induced paw licking response. The CRR significantly decreased the paw licking response at both early phase and late phase. However the observed effect is comparatively less to indomethacin treated group. Administration of CRS unable to inhibit formalin induced pain at both early phase and late phase of pain.

Mice, pre-treated with Pentazocine (20mg/kg) significantly increased TFL ($P<0.01$) after 30 minute and non-significantly at 60minutes onwards (Table - 4). CRR significantly increased TFL in time dependant manner i.e., $P<0.01$ at 90 min, $P<0.05$ at 120 min and $P<0.01$ at 180 minute after drug administration. In contrast to this CRS shows significant increase in TFL only at 180minutes in comparison to control group (Table-4).

Table 1: Effect on carrageenan induced paw edema.

Treatment	3 hours	% inhibition	6 hours	% inhibition
Control	57.47 ± 3.53	--	39.29 ± 1.81	--
CRR	30.25 ± 1.64[¥]	47.36 ↓	23.27 ± 2.88[#]	40.77 ↓
CRS	28.84 ± 4.09[¥]	50.00 ↓	23.48 ± 2.23[#]	40.23 ↓
Phenylbutazone (100mg/kg,po)	23.42 ± 2.82[¥]	60.00 ↓	23.61 ± 1.77[#]	40.00 ↓

Data: Mean ± SEM; ↓ – Decrease
[¥]One way ANOVA – F value 23.340; P<0.001: DMTT – P < 0.05 for root, stem, phenyl butazone Vs normal control.
[#]One way ANOVA – F value 12.687; P<0.001: DMTT – P < 0.05 for root, stem, phenyl butazone Vs normal control.

Table 2: Effect on formalin induced paw edema.

	Percentage increase in paw oedema			
Treatment	**24 hours**	**Percentage inhibition**	**48 hours**	**Percentage inhibition**
Control	43.62 ± 2.71	--	30.78 ± 2.36	--
CRR	42.88 ± 2.14	--	41.29 ± 2.12	34.14 ↑
CRS	27.32 ± 4.61[¥]	37.36 ↓	30.83 ± 2.15	--
Diclofenac sodium (5mg/kg, po)	22.82 ± 2.11[¥]	47.68 ↓	17.13 ± 1.86[#]	44.34 ↓

Data: Mean ± SEM; ↓ – Decrease
[¥]One way ANOVA – F value 11.0; P < 0.001: DMTT – P < 0.05 for stem and diclofenac sodium Vs normal control.
[#]One way ANOVA – F value 19.727; P < 0.001: DMTT – P < 0.05 for diclofenac sodium Vs normal control.

Table 3: Effect on formalin induced paw licking response.

Treatment	Number of paw lickings			
	0-10 min	% inhibition	20-30 min	% inhibition
Control	16.33 ± 0.989	--	15.83 ± 0.83	--
CRR	12.00 ± 1.48¥	26.51 ↓	11.66 ± 0.98#	26.34 ↓
CRS	13.50 ± 1.23	17.33 ↓	14.83 ± 1.13	06.31 ↓
Indomethacin (10mg/kg,po)	9.33 ± 0.71¥	42.86 ↓	7.33 ± 0.75#	54.00 ↓

Data: Mean ± SEM;↓ – Decrease
¥One way ANOVA – F value 6.565; P < 0.001: DMTT – P < 0.05 for root, Indomethacin Vs normal control.
#One way ANOVA – F value 16.837; P < 0.001: DMTT – P < 0.05 for root, Indomethacin Vs normal control

Table 4: Effect on tail flick response

Treatment	Initial TFL (sec.)	TFL after drug administration (sec.)					
		30 min	60 min	90 min	120 min	180 min	240 min
Control	3.63 ± 0.59	2.99 ± 0.45	2.53 ± 0.25	2.78 ± 0.21	2.70 ± 0.24	2.35 ± 0.14	2.93 ± 0.54
CRR	2.56 ± 0.18	03.01 ± 0.18	3.05 ± 0.14	4.05 ± 0.23**	4.26 ± 0.34*	4.10 ± 0.35**	3.90 ± 0.23
CRS	2.75 ± 0.13	2.83 ± 0.30	2.73 ± 0.15	2.76 ± 0.20	3.10 ± 0.08	3.28 ± 0.23*	3.16 ± 0.11
Pentazocine (20mg/kg, ip)	2.34 ± 0.23	5.97 ± 0.90**	3.51 ± 0.57	3.58 ± 0.37	2.98 ± 0.35	2.93 ± 0.33	2.73 ± 0.23

Data: Mean ± SEM; *P < 0.05, ** P <0.01 (unpaired t test)

DISCUSSION

Carrageenan induced inflammation in rats is one of the most suitable acute model to screen anti-inflammatory agents. The intraplantar infection of carrageenan in rats leads to paw edema. Its first phase (Up to 3 hours after injection of carrageenan) results from the concomitant release of mediators: histamine, serotonin and kinins on the vascular permeability. The second phase is correlated with leukotrienes[11]. In present study, both root and stem parts of test drug produced a considerable suppression of carrageenan induced paw oedema at both the phases. The inhibitory effect on carrageenan induced inflammation in the rats observed could be due to inhibition of enzyme cyclo oxygenase , leading to inhibition of prostaglandins.

The formalin-induced inflammation in the rats foot may be conveniently divided into two parts, the first involving 5-hydroxytryptamine as mediator and the second mediator which is unrelated to 5-hydroxytryptamine[12]. In contrast to results obtained in carrageenan induced paw oedema, root of test drug failed to suppress the paw oedema at both time intervals, where as stem part able to suppress oedema at on 24 hour after formalin injection. Thus shows root part is not having 5-HT suppression activity while stem is having capacity to suppress 5-HT mediated paw oedema.

The mechanism for testing analgesic was selected such that both centrally and peripherally mediated effects were investigated by adopting formalin induced pain and tail flick response methods. Formalin injection to plantar aponeurosis of rats shows pain response in two phases viz., initial and late phases. The initial phase lasts for 0-10 min. of formaldehyde injection, it is supposed to be mediated through modulation of neuropeptides[13]. The second phase, which is observed 20-30 minutes of formaldehyde injection, is supposed to be mediated through release of inflammatory mediators like prostaglandin etc. The root of test drug (CRR) significantly decreased the paw licking episodes at both the phases so as the indomethacin which is a non-selective cyclooxygenase inhibitor. This suggests that the antinociceptive effect of the CRR was mediated by both neurogenic and inflammatory mechanisms. In this model stem part of test drug (CRS) failed to antagonize the formalin induced algonic effect.

Tail flick model which is thermal induced nociception indicates narcotic involvement which is sensitive to opioid μ receptors[14]. The ability of the CRR to prolong the reaction latency to thermally induced pain in mice further suggests central analgesic activity. The effect observed with pentazocine was short lived where as the effect observed in CRR is long lasting. This indicates that the CRR exhibit analgesic effect by central action. The mechanism through which this effect is brought about may be due to modulation of opioid receptors or by release of endogenous analgesic factors like endorphin etc. Further CRS is also having TFL prolonging effect, but the magnitude of prolongation is comparatively less.

It has been reported that the plant *C.repanda* is having alkaloids, tannins, glycosides and flavonoids[15]. Presence of these phyto-constituents in *C rependa* supports the claim that these compounds have antinociceptive and anti-inflammatory properties since alkaloids, flavonoids and saponins have been found in other natural products with analgesic and anti-inflammatory properties[16, 17.].

CONCLUSION

From the present study, it can be conclude that, roots of *Cissus rependa* have significant anti-inflammatory and analgesic activity in comparison to stem. Hence, it can be preferred in the treatment of pain and inflammation. However studies to systematically extract the active metabolites from the roots will be needed to ascertain their analgesic and anti-inflammatory properties separately.

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Chemical Composition and Antibacterial Activity of Essential Oil from *Anisomeles* Species grown in India

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ABSTRACT

Anisomeles indica L., and *Anisomeles malabarica* L. R. Br. Ex Sims, growing wild in India. These shrubs become gives biological effect because of chemical composition of essential oil. Now it is interesting to know available chemicals in it, which also support the claim biological activities still, by the researchers. The chemical composition and antibacterial activity of the essential oils from *A. indica* and *A. malabarica* were investigated together here for the first time. The aerial parts (Stem, leaves, flowers and fruit) of hydrodistilled essential oils were analyzed by gas chromatography-mass spectrometry (GC-MS), and antibacterial activity was individually evaluated against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus pumilus* using a paper disc diffusion method. Collectively more than fourty compounds were identified in *A. indica* and *A. malabarica*, representing 98.29–97.88% of the total essential oil, respectively. The major constituents of essential oils obtained from the *A. indica*, were linalyl acetate (15.3%), and α-thujone (11.9%). The most abundant compounds in essential oils of *A. malabarica*, were - α-thujone (17.6%), terpenyl acetate (16.45%) and, *δ*-cadinene (11.5). All tested G+ ve & G-ve were inhibited by essential oil samples. The GC-MS results of both plants indicated the essential oil is rich in monoterpenes and terpenoids, which have been implicated antibacterial activity, comparable to gentamycin, it was used as a positive probe. The current findings also help to differentiate the valuable *Anisomeles* species for phyto-pharmaceuticals.

Key words: *Anisomeles indica* L., *Anisomeles malabarica* L.R.Br., antibacterial activity, GC-MS

INTRODUCTION

Even from ancient times, herbs and spices have been added to different types of food to improve the flavor and organoleptic properties.[1] Especially popular today is the concept of food that combines nutritional and medicinal benefits. Many natural compounds isolated from plants have demonstrated a wide spectrum of biological activities. Among these various kinds of natural substances, essential oils from aromatic and medicinal plants receive particular attention as potential natural agents for food preservation.[2] Moreover, essential oils are proven to have various pharmacological effects, such as spasmolytic, carminative, hepatoprotective, antiviral and anticarcinogenic effects.[3]

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The genus *Anisomeles* L. R. Br. belongs to the Lamiaceae family, and comprises over 20 species whose centre of distribution is located in the tropical Asia and Australia. These are annual or perennial semi-bushy aromatic plants that inhabit arid, sunny, stony and rocky regions. Many members of this genus are well known for their aromatic and medicinal character.[4] Three species occur in India yet, *Anisomeles indica, Anisomeles malabarica* and *Anisomeles heyneana*.[5] Out of these *A. indica* and *A. malabarica* were investigated for their Pharmacognostical and various biological activities yet.[6-7]

Anisomeles indica are used in folk medicine all over the India. It is popularly known as 'Jirnya' in northeastern part of India, where it receives widespread used as folk medicine, predominantly in the treatment of intestinal disorders and intermittent fever. *Anisomeles indica* have anti-microbial, astringent, carminative, ethanolic extract (50%) of the herb showed hypothermic activity and when burn acts as a mosquito repellant. The essential oil present in the herb is useful in uterine affections. And, *Anisomeles malabarica* useful in halitosis, epilepsy, hysteria, amentia, anorexia, dyspepsia, colic, flatulence, intestinal worms, fever arising from teething

children, intermittent fever, gout, swelling and diarrhea. Recently the valued plant investigated for its herbaceous activity.[8]

A literature survey reveals a reports on the GC-MS study of essential oil of *A. indica*, and none for *A. malabarica*.[9]

In the present work, we investigated the essential oil chemical composition of *A. indica* and *A. malabarica*. In addition, the aim of this study was to determine physical constant and to perform antibacterial activity of the isolated essential oils, which have been not reported still date.

MATERIALS AND METHODS

Plant material and reagents

Plant samples of the 2 species were collected from their type localities Toranmal (Maharashtra), and Dindigul (Tamilnadu); India. The collection was carried out two times, mansoon & autumn season, to accurately reflect the chemical composition of the respective plants. The identity of the plant material was verified by Prof. (Dr.) H.B Singh, Head, Raw Materials Herbarium and Museum, NISCAIR, New Delhi, India. Voucher specimen number HNSIPER/Herb-03 of *A. indica* & HNSIPER/Herb-04 of *A. malabarica* was deposited at the Institute level.

All applied reagents were of the highest purity available and purchased from the Sigma–Aldrich Chemical Company.

Isolation of essential oil

The powder of aerial part (flower, leaves, and stem) of *Anisomeles indica* (AIA) and *Anisomeles malabarica* (AMA) was prepared by passing through sieve # 44, and kept in tightly closed polyethylene bags. Air-dried plant material of each was subjected to hydro- distillation for 2 h with a Clevenger-type apparatus, and then dried over anhydrous sodium sulphate. The oil was stored at 4 °C in a sealed brown vial until analysis.

Microbial strains

The essential oils from AIA and AMA were individually tested against four pathogenic bacterias: *Escherichia coli* NCIM 2109, *Pseudomonas aeruginosa* NCIM 2036, *Bacillus pumilus* NCIM 2327, and *Staphylococcus aureus* NCIM 2079. All the bacterial strains were grown and maintained on nutrient agar slants. Bacterial strains were kindly supplied by stock cultures from Dept. of Biosciences, Saurastra University (Rajkot, India).

Gas chromatography/mass spectrometry analysis of essential oil

The GC (Shimadzu GCMS Q.P. 2010^{TM}) system coupled to Shimadzu Turbo Mass MS. Shimadzu GCMS Q.P. 2010^{TM} 30 m x 0.25 mm x 0.25 µm BPX-5 (SGE) column was used with helium as the carrier gas 1ml/min. The oven program was kept at 50 ^{0}c for 10 min, programmed to reach 325^0 C at a rate of 5 ^{0}C / min, and 1 µl injection (split 1:10) at 280 ^{0}C were made. Mass spectra were recorded at 70 eV. Mass range was m/z 40 to 250.

The essential oil diluted with chloroform and then injected in column. The quantification of the components was performed on the basis of their GC peak areas on the column.[10] Identification of the oil components was based on their retention indices determined by reference to a homologous series of *n*-alkanes, and by comparison of their mass spectral fragmentation patterns with those reported in the literature, and stored on the MS library [NIST database 98/ NBS 75K]. The percentages of each component are reported as raw percentages based on total ion current without standardization.

Physical evaluations

The volatile oils individually evaluated to determine their, organoleptic character, percentage volatile oil content, density and, Refractive index.[11]

Antibacterial screening

Antibacterial activity of essential oils was tested by the paper disc diffusion method according to the slightly modified National Committee for Clinical Laboratory Standards Guidelines (NCCLS, 2001) using 100 µl of suspension of the tested microorganisms, containing 2.0x10^6 colony forming units (cfu/ml). Mueller-Hinton agar (15 ml), sterilized in a flask and cooled to 45–50 ^{o}C, was distributed to sterilized Petri dishes with a diameter of 9 cm. The filter paper discs (6 mm in diameter, Whatman No. 1) were individually impregnated with 10 µl of the sample dissolved in dimethylsulfoxide (DMSO), which was subsequently placed on the surface of the inoculated Petri dishes. The essential oils concentrations in DMSO were adjusted to 3.0 mg/ml. The Petri dishes were kept at 4 ^{o}C for 2 h, and then incubated at 37 ^{o}C for 24 h. The diameters of the inhibition zones were measured in millimeters. Controls were set up with equivalent quantities of DMSO. Studies were performed in triplicate, and the developing inhibition zones were compared with those of reference discs. Antibiotic gentamycin (30 µg) was used as reference.

RESULTS

GC-MS analysis

The essential oils of *A. indica* and *A. malabarica* were subjected to detailed GC-MS analysis in order to determine their volatile constituents. Exactly 41 compounds were identified in two samples are given in Table 1.

In AIAEO (Essential oil of *A. indica*), 36 compounds were identified, representing 98.29% of the total essential oil. The most abundant components were oxygenated monoterpene (26.10%) and oxygenated sesquiterpenes (44.86%). The high percentages of linalyl acetate (15.3%) and, α-thujone (11.9%) proved that this essential oil clearly belongs to the mixed chemotype. In contrast, the essential oil obtained from plant material of *A. malabarica* (AMAEO),

Table 1: The percentage concentration of compounds found in essential oils obtained from A. indica and A. malabarica

		Content (%) in	
RI[a]	Compound	AIAEO	AMAEO
931	α-pinene	1.2	2.4
946	Camphene	1.5	0.7
975	β-pinene	3.35	tr[b]
995	3-Octanol	-	0.6
1030	1,8-Cineole	tr	0.1
1066	cis-sabinene hydrate	0.1	1.7
1098	Linalool	0.1	1.1
1121	*cis-p*-menth-2,8-en-1-ol	0.2	0.2
1147	Camphor	6.5	4.7
1168	Borneol	2.9	2.9
1198	Myrtenol	tr	tr
1205	α-thujone	11.9	17.6
1210	Linalyl acetate	15.3	-
1229	Nerol	tr	tr
1235	Methyl ether Thymol	tr	tr
1258	Geraniol	0.1	0.1
1266	Geranial	tr	tr
1292	Thymol	1.8	3.8
1320	Bornyl acetate	-	0.37
1321	Isobornyl formate	5.7	1.45
1328	Terpenyl acetate	-	16.45
1335	Anisole	1.38	0.5
1337	2-Isopropylbenzaldehyde	1.38	0.5
1360	Eugenol	1.05	3.55
1488	*n*-Nonanyl acetate	tr	1.1
1536	δ-cadinene	-	11.5
1551	Caryophyllene	2.32	0.92
1553	Isocaryophyllene	-	0.31
1819	Caryophyllene oxide	2.86	7.81
1837	Epiglobulol	tr	1.25
1838	Globulol	tr	tr
1864	trans - Naphthalene	tr	tr
1864	Trifluoroacetyl - isomenthol	0.1	-
1947	Nerolidyl acetate	0.63	0.2
2122	Farnesyl acetone	4.89	9.98

Table 1: *continued*

RI[a]	Compound	Content (%) in AIAEO	Content (%) in AMAEO
2390	α-Bisabolol	5.85	2.75
2463	*trans*-Phytol	7.1	3.23
2464	Citronellol	1.1	1.35
2465	1,3 trans Menthol	tr	2.45
2465	Isomenthol	tr	tr
2646	Azulene	-	1.63
	Monoterpene hydrocarbons	14.40	19.47
	Oxygenated monoterpenes	26.10	23.44
	Sesquiterpene hydrocarbons	5.84	8.46
	Oxygenated sesquiterpenes	44.86	38.46
	Diterpene hydrocarbon	07.09	08.05
	Total identified	**98.29**	**97.88**

[a]Temperature program Kovat's retention index. [b]tr: Trace amount (< 0.1%).

was characterized by a high content of oxygenated sesquiterpenes (38.46%) and oxygenated monoterpenes (23.44%), with α-thujone (17.6%), terpenyl acetate (16.45%) and, *δ*-cadinene (11.5) as the main constituents. Thirty nine compounds were identified, representing the 97.88% of the total essential oil content.

Physical evaluations

The results of physical evaluation of essential oils are dipected in Table 2.

Antimicrobial screening

The antimicrobial activities of *A. indica* and *A. malabarica* essential oils were evaluated by a paper disc diffusion method against G+ve, and G-ve bacteria. Essential oils exhibited antibacterial activity against the tested strains, but in variable degree. Results are comparable to the antibiotic gentamycin, used as a positive probe (Table 3).

Table 2: Physical evaluation of volatile oil from A. indica and A. malabarica

Parameter	*A. indica*	*A. malabarica*
Organoleptic Character		
Color	Pale yellow	Pale yellow
Odor	Warm & Woody	Warm & terpeny
Taste	Slightly Pungent then spicy	Pleasant with spicy
Density	0.9666	0.9910
Refractive Index	1.4939	1.4897
% Volatile oil (w/w)	0.06	0.05

Table 3: Inhibition zones (mm) of the A. indica and A. malabarica essential oils

Micro-organisms	*A. indica** (10µl/disc) Mean ± SD‡	*A. malabarica †* (10µl/disc) Mean ± SD‡	Gentamycin (10µl/disc) Mean ± SD‡
Staphylococcus aureus NCIM 2079	14 ± 0.023	10 ± 0.028	41 ± 0.026
Bacillus pumilus NCIM 2327	13+0.013	11 ± 0.013	33 ± 0.018
Escherichia coli NCIM 2109	10 ± 0.027	08 ± 0.023	16 ± 0.005
Pseudomonas aeruginosa NCIM 2036	10 ± 0.019	10 ± 0.003	13 ± 0.006

*A. indica - collection at Toranmal (mansoon & autumn); †A. malabarica - collection at Dindigul (mansoon & autumn); ‡Standard deviation of three readings

CONCLUSION

The GC-MS results of both plants indicated the essential oil is rich in terpenes and terpenoids which have been implicated in plant's pharmacological activities. The results found in antibacterial activity, is because of monoterpenes & terpenoids presenet in essential oils. Linalyl acetate, α-thujone *δ*-cadinene, and terpenyl acetate are responsible for bacterial sensitivity. The data indicated that Gram-positive *B. pumilus* was the most sensitive strain tested to the oils of *A. indica* and *A. malbarica*. Gram-negative *P. aeruginosa* is known to have a high level of intrinsic resistance to virtually all known antimicrobials and antibiotics, due to a very restrictive outer membrane barrier, highly resistant even to synthetic drugs.[12] However, *A. indica* inhibit growth of this bacterium. Confirming previous reports, it was found that the strength and spectrum of activity varied between

investigated *Anisomeles* species and Gram-positive bacteria were generally more sensitive to the effects of the oils.

Although essential oils of *A. indica* and *A. malabarica* have significant differences in their chemical compositions, showed very effective antibacterial activities. The results of this study suggest the possibility of using the essential oil of these two *Anisomeles* species as natural food preservatives, and potential sources of antibacterial ingredients for the food and pharmaceutical industry. Our results suggest that the essential oils of those species may warrant further investigation for their potential therapeutic efficacy.

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Antimicrobial Potential of *Eupatorium adenophorum* Spreng

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ABSTRACT

The antimicrobial activity of Petroleum-ether, Benzene, Chloroform, methanol and aqueous extracts of crude leaves of *Eupatorium adenophorum* Spreng were tested against the growth of *Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Escherichia coli, Klebsiella aerogenes* and *Pseudomonas aeruginosa* by Paper disk diffusion technique. Nearly all the extracts were found to exhibit moderate to good antibacterial activity against the bacterial pathogens tested and the petroleum ether extract recorded largest zone of inhibition against *B.subtilis*. The positive results so obtained were compared with that of the reference standard antibiotic ciprofloxacin. Antifungal activity of the above extracts were tested on fungal strains *Aspergilus niger, Aspergilus candidus* and *Candida albicans* using Fluconazole as the standard drug. The result shows significant antimicrobial activity of extracts against tested fungi and bacteria.

Keywords: *Eupatorium adenophorum* Spreng, Antibacterial, Antifungal, Extracts.

INTRODUCTION

Infectious diseases are caused by pathogenic microorganisms, such as bacteria, viruses, parasites or fungi. Diseases can spread, directly or indirectly, from one person to another. Infectious diseases are the second leading cause of death worldwide. About one – fourth of all the medicines we use, come from rainforest plants. However, scientific have been conducted only to a limited extent with few medicinal plants[1,2]. The present study was designed to search for newer, safer and more potent antimicrobials components which may accomplish our present need. Herbal medicines have received much attention as a source of new antibacterial drugs since they are considered as time tested and comparatively safe both for human used and for environment. Aromatic and medicinal plants are known to produce certain bioactive molecules which react with other organisms in the environment, inhibiting bacterial or fungal growth[3,4].

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Eupatorium Linn (Fam Asteraceae) is a large genus of herbs, shrubs or undershrubs, distributed chiefly in tropical America, a few species occurring in Europe, Africa and Asia and India. Different parts of *Eupatorium adenophorum* Spreng are used in Ayurveda and other folk medicines for the treatment of cut and wounds. Leaves are used as an application to unhealthy ulcers. A decoction of the plant and the juice of the leaves are traditionally used as popular haemostatic remedy for various kinds of hemorrhage. Traditionally the leaves paste mix with mustard oil is useful for ulcer. Every part of the plant either alone or in combination has also been recommended for snake bite. *Eupatorium* adenophorum Spreng consist of various bioactive constituents like triterpenoids, flavanoides, sterols, saponins, triterpene alcohols and lactones[5-8].

MATERIALS AND METHODS

Collection and Authentication of Plant

Leaf samples of *E. adenophorum* Spreng were procured from the forest of Nagdhar Pokhari Chamoli (Uttarakhand) and identified Botanical Survey of India, Northern Regional centre, Dehradun (BSD) with the Accession number 1127802, 1127803. A voucher specimen has been preserved in the department for further verification.

Preparation of Plant Extracts

The air dried leaves of *E. adenophorum* Spreng as moderately coarse powders were completely extracted with petroleum ether (60-80), benzene, chloroform, alcohol (95% v/v) and water in soxhlet extractor apparatus. The extracts were concentrated under vacuum (50^0), dried and weighed. Percentage extractives by successive extraction were found to be 2.390, 1.029, 1.179, 2.189, 3.456 and 3.968 percent (w/w) respectively. These extracts were dissolved in Dimethyl sulfoxide (DMSO) containing 1000 µg/ml.

Microbial strains

In total 9 microbial strains used for the experiment were collected as pure cultures from I.F.T.M, Department of Microbiology, Moradabad, UP. Both gram positive, gram negative bacteria and fungi were taken for the test. The microorganisms were maintained on nutrient agar medium (HIMEDIA).

Antimicrobial Activity

Antibacterial and Antifungal activity of the crude extract was investigated against 9 bacterial and fungal strains by the paper disk diffusion technique[9] using 100µl of suspension containing 108 CFU/ml of bacteria spread on nutrient agar medium. Sterile 6mm diameter filter paper discs were impregnated with 400µg of each of the sterile test material (petroleum ether, benzene, chloroform, methanol and aqueous extract) and placed in nutrient agar medium. Ciprofloxacin (30µg/disc) disc were used as positive control to ensure the activity of standard antibiotic against the test organisms. The sample discs and the standard antibiotic discs were placed gently on the previously marked zones in the agar plates pre- inoculated with the test bacteria and fungi. The plates were than kept in a refrigerator at 4^0c for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were than inverted and kept in an incubator at 37^0c for 24 hours. The antimicrobial potency of the test agents were measured by their activity to prevent the growth of the microorganisms surrounding the discs which gave clear zones of inhibition. After incubation, the antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale[10].

Minimum Inhibitory Concentration

MIC was determined using the broth dilution technique.[11,12] The minimum inhibitory concentration value was determined for the microorganisms that were sensitive to the extracts under study. A two- fold serial dilution of each extracts was made to using nutrient broth. MIC is defined as the lowest concentration where no visible turbidity was observed in the test tubes.

RESULTS AND DISCUSSION

Preliminary antibacterial studies were conducted on dried plant extracts of *E. adenphorum* Spreng using *Bacillus subtilis* (ATCC-6633), *Bacillus cereus (ATCC 11778) Staphylococcus aureus (ATCC 25923), Escherichia coli (ATCC 11229), Klebsiella aerogenes (NCTC 418) and Pseudomonas aeruginosa* (ATCC 35032) at concentration of 100µl/disc by disc diffusion method. The significant antibacterial activity was determined by measuring the diameter of zone of inhibition and compared with the standard drug ciprofloxacin. From the data Table 1, it was found that all the crude extracts (400µgm/disc) exhibited moderate to good antibacterial activity against the bacterial pathogens tested herein and the petroleum ether extract of *E. adenophorum* Spreng recorded largest zone of inhibition (17 and 15 mm in diameter) against *B.subtilis* and *E.* coli. Antimicrobial antibiotic ciprofloxacin (30µgm/disc) was found to be active against all the bacteria tested herein. From the results of

Table 1: Antibacterial activity of crude leaf extracts from *E. adenophorum* Spreng

Bacteria	Diameter of zone of inhibition(mm) (Crude extract 400µ gm/disc)					Ciprofloxacin (30µ gm/disc)
	PEE	CE	BE	AE	Aq.E	
Gram positive						
B.subtilis	17	12	12			
B.cerus	12	11	11	14	13	22
S.aureus	11	11	11	15	10	20
				13	12	17
Gram negative						
E.coli	15	13	11	13	14	19
K. aerogenes	11	10	10	12	10	17
P.aeruginosa	12	10	10	12	10	16
DMSO	-	-	-	-	-	-

the MIC presented in Table 2 the petroleum ether extracts exhibited the lowest MIC value (120 µ gm / ml) against *B. subtilis* and the chloroform extracts of *E. adenophorum* Spreng exhibited the lowest MIC value (120 µ gm/ml) against *E.coli*.

The result of antifungal activity observed and reported in Table 3 and Table 4. The antifungal activity of dried plant extracts was done by disc diffusion method against two typical pathogen namely *Candida albicans* (ATCC 10231), *Aspergillus niger* (ATCC 16404) and *Aspergillus candidus* (NCIM 883) using Fluconazole as standard drug. The effect of the different extracts of *E. adenophorum* Spreng on the pathogenic fungi was carried out at concentration (400µgm/disc) at 25±2°C for 48 hrs of incubation. The extracts were found to have moderate antifungal activity. The petroleum ether extract shows largest zone of inhibition (16 mm in diameter) against *Candida albicans*. Antifungal antibiotic Fluconazole (30µgm/ml) was also found to be active against all the fungal species tested. The petroleum ether and alcoholic extracts exhibited the lowest MIC value (250µgm/ml) against *Candida albicans*.

This study confirmed that the petroleum ether extract (PEE) of leaves of *E.adenophorum* Spreng exhibit antimicrobial activity and the effects are attributable due to the presence of triterpenoids in the plant.

In conclusion, the fact that the extracts (PEE and AE) produced inhibitory activities but less when compared to reference drugs against almost all the test bacteria and fungi provides some scientific basis for some of the uses in traditional medicine like treatment of boils and scabies and as antiseptic. We therefore suggest the isolation and possible characterization of the active constituent(s) from the extracts of this plant species as possible antibacterial agents.

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Table 2: MIC of crude leaf extracts from *E. adenophorum* Spreng

	MIC(Crude extract µ gm/ml medium)					
Bacteria	**PEE**	**CE**	**BE**	**AE**	**Aq.E**	**Ciprofloxacin**
B.subtilis	120	250	500	250	500	5
B.cerus	250	500	750	250	500	5
S.aureus	800	820	750	750	800	5
E.coli	500	120	500	500	250	5
K. aerogenes	250	250	800	250	750	5
P.aeruginosa	250	500	500	250	500	5

Key: PEE = Petroleum ether extract, CE = Chloroform extract, BE = Benzene extract, AE = Alcoholic extract, Aq.E = Aqueous extract, B.subtilis = Bacillus subtilis, E.coli = Escherichia coli, S.aureus = Staphylococcus aureus, - = No growth

Table 3: Antifungal activity of crude leaf extracts from *E. adenophorum* Spreng

	Diameter of zone of inhibition(mm) (Crude extract 400µgm/disc)					Fluconazole (30µgm/disc)
Fungi	**PEE**	**CE**	**BE**	**AE**	**Aq.E**	
C. albicans	16	12	12	15	13	20
A. niger	13	11	12	13	11	16
A. candidus	13	12	11	13	10	18
DMSO	-	-	-	-	-	-

Table 4: MIC of crude leaf extracts from *E. adenophorum* Spreng against fungi.

	MIC(Crude extract µ/ml medium)					
Fungi	**PEE**	**CE**	**BE**	**AE**	**Aq.E**	**Fluconazole**
C. albicans	250	750	750	250	500	5
A. niger	500	800	820	500	850	5
A. candidus	500	750	1000	500	1000	5

Key: PEE = Petroleum ether extract, CE = Chloroform extract, BE = Benzene extract, AE = Alcoholic extract, Aq.E = Aqueous extract, MIC = Minimum inhibitory concentration, - = No growth.

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Antibacterial, Insecticidal and Free radical scavenging activity of methanol extract of *Ziziphus rugosa* Lam. (Rhamnaceae) fruit pericarp

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ABSTRACT

Introduction: *Ziziphus rugosa* Lam. belongs to the family Rhamnaceae and is found chiefly in deciduous and semi-evergreen forest of Western Ghats. The present study was undertaken to determine antibacterial, insecticidal and free radical scavenging activity of methanol extract of *Ziziphus rugosa* Lam. fruit pericarp. **Methods:** The powdered fruit pericarp of *Z. rugosa* was extracted with methanol. Antibacterial activity of methanol extract was determined against *Escherichia coli* and *Staphylococcus aureus* by Agar well diffusion method. Free radical scavenging activity was determined using DPPH assay. The insecticidal activity of extract was tested against second instar larvae of *Aedes aegypti*. **Results:** The extract exhibited dose dependent inhibition of test bacteria. Among bacteria, *E. coli* was found to be more susceptible to extract than *S. aureus*. All the concentrations of extract produced over 50% mortality of larvae and the larvicidal effect was found to be dose dependent. The extract caused 100% mortality of larvae at concentration of 50 mg/ml. The extract exhibited concentration dependent radical scavenging activity with an IC_{50} value of 61.88 µg/ml. The phytochemical analysis of extract showed the presence of alkaloids, saponins, flavonoids and glycosides. **Conclusion:** The extract, in suitable form, may be used to control bacterial diseases, free radical damage and arboviral diseases. The phytoconstituents present in the extract may be responsible for the tested biological efficacies of extract. Further studies on isolation of active constituents from the extract and their biological activity are under investigation.

Key words: *Ziziphus rugosa* Lam., Agar well diffusion, Free radical scavenging activity, DPPH assay, Insecticidal activity, *Aedes aegypti*

INTRODUCTION

Ziziphus rugosa Lam. belongs to the family Rhamnaceae. It is a large straggling scandent armed shrub with large elliptic usually subcordate leaves, paniculate flowers and wood is reddish, moderately hard and fruit is small drupe, glabrous, white when ripe. The plant is found chiefly in deciduous and semi-evergreen forest of Western Ghats and is commonly called as suran in Hindi, chunu koli in Urdu and Badara in Sanskrit and in local language is called as belamarluhanu. Bark is astringent and antidiarrhoeal. Flowers are used in prescriptions for menorrhagia. Stem and fruit are hypotensive. The bark contains vanillic acid, betulin, betulinic acid, kaempferol, quercetin, myricetin, apigenin and apigenin-7-O-glucoside. The bark also contains several N-formyl cyclopeptide alkaloids. The triterpene saponins isolated from the bark showed CNS depressant, tranquilizing and analgesic activity in albino rats and produced no hepatotoxicity. The cyclopeptide alkaloids of the plant show antibacterial as well as antifungal activity.[1,2] The present study was undertaken to determine antibacterial, insecticidal and free radical scavenging activity of methanol extract of fruit pericarp of *Z. rugosa*.

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MATERIALS AND METHODS

Collection and identification

The fruits of *Z. rugosa* were collected from the Doddabetta forest range (located between 12°49'N and 75°57'E longitude) of Sakaleshpura, Hassan district, Karnataka and authenticated by Prof. K.G. Bhat, Udupi, Karnataka. The voucher specimen (KU/AB/KSV/75) deposited in the department of Botany, Jnanasahyadri, Shankaraghatta-577451, Karnataka for future reference.

Extraction and Phytochemical analysis

For extraction, about 50 g of the dried and powdered seed material was taken and added to 100 ml of methanol. The mixture was sonicated for 30 min and then left at room temperature overnight. The extracts were filtered over Whatman No 1 filter paper and the filtrates were concentrated under reduced pressure to pasty mass. The methanol extract was subjected to chemical tests to screen the presence of various secondary metabolites.[3,4]

Antibacterial activity of methanol extract

The antibacterial efficacy of methanol extract of fruit pericarp was tested against bacteria namely *Staphylococcus aureus* MTCC-902 and *Escherichia coli* MTCC-405 obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India by Agar well diffusion method.[5] In this method, 24 hours old Nutrient broth cultures of test bacteria were swabbed uniformly on solidified sterile Nutrient agar plates using sterile cotton swab. Then, aseptically wells of 6 mm diameter were bored in the inoculated plates with the help of gel puncher and the different concentrations of extract (5, 10, 25 and 50 mg/ml of 10% DMSO), Standard (Chloramphenicol 1 mg/ml) and Control (10% DMSO) were added into the respectively labeled wells. The plates were incubated at 37°C for 24 hours in upright position. The experiment was carried in triplicates and the zone of inhibition was recorded.

Insecticidal activity of methanol extract

For determining insecticidal activity, the extract was dissolved in 10% Dimethyl sulfoxide (DMSO) to get different concentrations of extract namely 5, 10, 25 and 50 mg/ml. The insecticidal efficacy of methanol extract was determined against second instar larvae of *Aedes aegypti*. Twenty larvae were placed separately into beakers containing different concentrations of extract. A beaker containing DMSO without extract serves as control. The larvicidal effect of extracts was determined by counting the number of dead larvae after 24 hours, 48 hours and 72 hours. The test was repeated thrice and the percentage of larval mortality was calculated.[6]

Free radical scavenging activity of methanol extract

The antioxidant activity, in terms of radical scavenging ability, of different concentrations of methanol extract and the standard (Ascorbic acid) was tested on the basis of the radical scavenging effect of the stable 1,1-diphenyl-2-picryl-hydrazyl (DPPH)-free radical activity.[7] Different concentrations namely 25, 50, 100, 250 and 500 µg/ml of methanol extract and standard were prepared in methanol. 0.002% of DPPH was prepared in methanol. In clean and labeled test tubes, 2 ml of DPPH solution was mixed with 2 ml of different concentrations of solvent extracts and standard separately. The tubes were incubated at room temperature in dark for 30 minutes and the optical density was measured at 517 nm using UV-Vis Spectrophotometer. The absorbance of the DPPH control (containing no sample) was also noted. The degree of stable DPPH* decolorization to DPPHH (reduced form of DPPH) yellow indicated the scavenging efficiency of the extract. The scavenging activity of the extract against the stable DPPH* was calculated using equation: Scavenging activity in % = A – B / A × 100 [Where A is the absorbance of control and B is the absorbance of test/standard].

RESULTS

The preliminary phytochemical analysis of methanol extract of fruit pericarp showed the presence of alkaloids, saponins, flavonoids and glycosides. The result of antibacterial activity of methanol extract of *Z. rugosa* fruit pericarp is presented in Table 1. Results were recorded as presence or absence of zones of inhibition around the well. The inhibitory zone around the well indicated the absence of bacterial growth and it as reported as positive and absence of zone as negative. The extract exhibited dose dependent inhibition of test bacteria. Among bacteria, *E. coli* was found to be more susceptible to extract than *S. aureus* as revealed by wider zones of inhibition. The inhibition caused by standard was found to be higher than all the extract concentrations. Control did not reveal any inhibition of test bacteria.

Table 1: Antibacterial activity of methanol extract of *Z. rugosa* fruit pericarp

Treatment	Concentration	Zone of inhibition in mm	
		E. coli	*S. aureus*
Methanol extract	5 mg/ml	14	11
	10 mg/ml	22	16
	25 mg/ml	24	20
	50 mg/ml	26	24
Standard	1 mg/ml	28	27
Control	10%	-	-

Table 2: Insecticidal activity of methanol extract of *Z. rugosa* fruit pericarp

concentration (mg/ml)	Total no. of larvae	No. of dead larvae	% mortality of larvae
0 (Control)	20	00	00.00
5	20	10	50.00
10	20	15	75.00
25	20	18	90.00
50	20	20	100.00

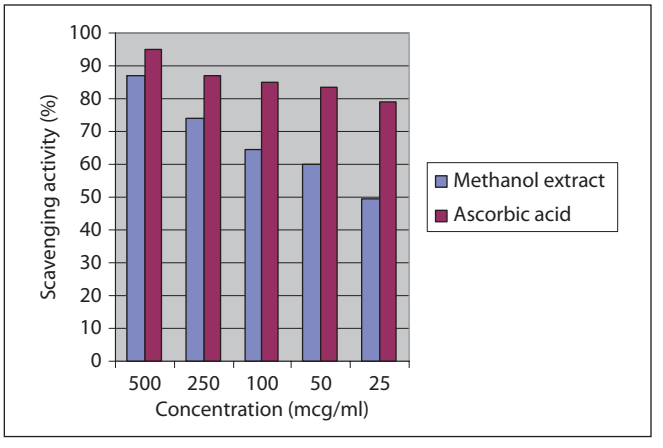

Figure 1: Free radical scavenging activity of methanol extract of *Z. rugosa* fruit pericarp

Insecticidal activity, in terms of larvicidal effect, of methanol extract on second instar larvae of *A. aegypti* is shown in Table 2. The larvicidal effect of extract was determined after 24 hours. Dead larvae were identified when they failed to move after probing with a needle in siphon or cervical region. All the concentrations of extract tested produced over 50% mortality of larvae. The methanol extract caused 100% mortality of larvae at concentration of 50 mg/ml.

The antioxidant activity, in terms of free radical scavenging activity, of different concentrations of seed extract is shown in Figure 1. The extract exhibited marked antioxidant activity by scavenging DPPH* (free radical) and converting into DPPHH. The extract exhibited concentration dependent radical scavenging activity i.e., higher the concentration, more scavenging potential. The methanol extract was able to reduce the stable free radical DPPH to the yellow colored diphenylpicrylhydrazine with an IC_{50} value of 61.88 µg/ml. The scavenging activity of ascorbic acid was greater than extract.

DISCUSSION

The plant *Z. rugosa* has been worked out for novel medical important compounds. Rugosanine-A, a cyclopeptide alkaloid, has been isolated from the stem bark of *Z. rugosa*.[8] A new glycoside zizyphoside has been isolated along with the betulic oleanolic, alphitolic and 2-α-hydroxy xyrusolic acids; zizyphoside on hydrolysis yielded altered aglycone, ebelin lactone.[9] Three flavonolds - kaempferol-4'-methylether, luteolin and luteolin-7-O-glucoside have been isolated from the barks of *Z. rugosa* and their structures were established by spectral evidences.[10] The flowers of *Z. rugosa* are extensively used for the treatment of hemorrhage and menorrhea. Fruit is edible and it also used to treatment of rheumatism and the decoction of the bark is used to heal the wounds and used for diarrhea.[2] The methanol extract of *Z. rugosa* bark showed significant antibacterial activity against *Streptococcus pyogens, Staphylococcus aureus* and *Pseudomonas aerogenes* whereas the methanol extract of leaves demonstrated moderate activity against *Salmonella typhi*. The chloroform extracts of the barks and leaves of *Z. rugosa* also showed antifungal activity. The methanol and ethyl acetate extracts of the bark of *Z. rugosa* revealed significant b-glucuronidase inhibitory activity. Lupeol, betuline, betulinaldehyde and betulinic acid, isolated from *Z. rugosa*, also showed good activity against a few bacteria.[11]

In many developing countries about 80% of available drugs come from medicinal plants and in industrialized countries plants make up the raw material for processes, which synthesize pure chemical derivatives.[12] The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds.[13] Plant derived products have received attention in recent years due to their diverse pharmacological activities.[14] Antimicrobial activity of tannins,[15] flavonoids,[16] saponins,[17] terpenoids[18] alkaloids[19] have been documented. In the present study, phytoconstituents namely alkaloids, saponins and flavonoids were detected in the extract which may account for the antibacterial activity.

Free radicals are found to be a product of normal metabolism. Although oxygen is essential for aerobic forms of life, oxygen metabolites are highly toxic. As a consequence, reactive oxygen species (ROS) are known to be implicated in many cell disorders and in the development of many diseases including cardiovascular diseases, atherosclerosis, chronic inflammation etc.[20,21] Although organisms have endogenous antioxidant defences produced during normal cell aerobic respiration against ROS, other antioxidants are taken both from natural and synthetic origin.[22] Antioxidants that can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, therefore, appear to be very important.[23] Synthetic antioxidants are widely used but their use is being restricted nowadays because of their toxic and carcinogenic effects. Thus, interest in finding natural antioxidants, without any undesirable effect, has increased greatly.[22] There are several methods available to assess

antioxidant activity of compounds. An easy, rapid and sensitive method for the antioxidant screening of plant extracts is free radical scavenging assay using 1,1, diphenyl-2-picryl hydrazyl (DPPH) stable radical spectrophotometrically. The hydrogen atom- or electron donating ability of extract and some pure compounds were measured from the bleaching of the purple colored methanol solution of DPPH. In presence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases.[24] DPPH is relatively stable nitrogen centred free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule.[25] DPPH radicals react with suitable reducing agents as a result of which the electrons become paired off forming the corresponding hydrazine. The solution therefore loses color stoichometrically depending on the number of electrons taken up.[26] Though the DPPH radical scavenging abilities of the extracts were less than that of ascorbic acid, the study showed that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. Thus, the methanolic extract of fruit pericarp can be a potential candidate to be explored for the treatment of damage caused by free radicals.

Mosquitoes are considered as a major health menace as they serve as disease transmitting vectors to humans and animals. Several mosquito species belonging to the genera *Anopheles*, *Aedes* and *Culex* are vectors of pathogens of various diseases such as malaria, filariasis, Japanese encephalitis, dengue, chickungunya etc. The approach to combat these diseases largely relied on interruption of the disease transmission cycle by either targeting the mosquito larvae through spraying of stagnant water breeding sites or by killing the adult mosquitoes using insecticides. The control of mosquito borne diseases is becoming difficult nowadays due to increasing resistance to pesticides, lack of vaccines and drugs to treat diseases transmitted by them. Hence, an alternative approach to control mosquitoes is the use of plant extracts. Search for natural insecticides, which are easily degradable and do not have any ill effects on the non-target population, remains one of the top priority issues for many countries. It is observed that the carbohydrates, saponins, phytosterols, phenols, flavonoids and tannins are having mosquito larvicidal activity. Prenylated xanthones, tetracyclic phenols and saponins are reported to be effective in controlling mosquito *A. aegypti*, the vector of yellow fever.[6, 27-30] In this study, the crude extracts have exhibited potent activity in terms of causing mortality of larvae. The presence of phytoconstituents such as saponins, flavonoids and others were detected in this study which might be responsible for the mortality of larvae. The death of larvae was observed within short period of time and thus could be used to control mosquito vectors and diseases transmitted by them.

CONCLUSION

The phytoconstituents present in the extract might be responsible for the tested biological efficacies of extract. The extract, in suitable form, could be used against bacterial diseases, free radical damage and arboviral diseases like chickungunya, dengue etc. Further studies on isolation of active constituents from the extract and their biological activity are under investigation.

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Anthocephalus Cadamba: A Review

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ABSTRACT

Anthocephalus cadamba is one of such ayurvedic remedy that has been mentioned in many Indian medicinal literatures. This article discusses about the medicinal values of *Anthocephalus cadamba*. In this communication, we reviewed the phytochemistry of *Anthocephalus cadamba* and its application in the treatment of various ailments like diabetes mellitus, diarrhoea, fever, inflammation, haemoptysis, cough, vomiting, wounds, ulcers, debility and antimicrobial activity. The major constituents of the plant are triterpenes, triterpenoid glycosides, flavanoids, saponins, indole alkaloids; cadambine, cadamine, isocadambine, isodihydrocadambine. This review discusses the investigations made by various workers related to chemical constituents, pharmacological action and toxicological studies of this plant since years till date.

Key words: *Anthocephalus cadamba*, Indole alkaloids, Pharmacological action, Antimicrobial action and Toxicological studies.

INTRODUCTION

Anthocephalus cadamba Miq., Syn. *A. indicus, A. rich, A.chiensis* (Lam.) Rich. Ex. Walp, *Neolamarckia cadamba* (Roxb.) Bosser. (Family-Rubiaceae) commonly called kadamba enjoys a hallowed position in Ayurveda- an Indian indigenous system of medicine. It is also named as Kadam. Other vernacular names of *Anthocephalus cadamba* have been listed in the Table 1. The tree is a medium to large sized deciduous tree attaining a height of 20-40 m and a girth of about 2-2.5 m with clean cylindrical branches and rounded crown. It is frequently found all over the India on the slopes of evergreen forests up to 500 m. It is found in the sub-himalayan tract from Nepal eastwards on the lower hills of Darjeeling terai in West Bengal where it is common; in Chota Nagpur (Bihar), Orissa and Andhra Pradesh, in the Andamans, it is very common in damp places along large streams, and in Karnataka and Kerala on the west coast, and western ghats at low level in wet places. It is also distributed in Thailand and Indo-china and eastward in Malaysian archipelago to Papua New Guinea.[1, 2] The bark of the plant is reported to possess tonic, bitter, pungent, sweet, acrid, astringent, febrifugal, anti-inflammatory, digestive, carminative, diuretic, expectorant, constipating and antiemetic properties and is given to treat the fever and inflammation of eyes. The flowers are used as vegetable. The leaves are slightly aromatic with unpleasant taste but the decoction of leaves good for ulcers, wounds, and metorrhea. Additionally, it is useful in the treatment of snake-bite. It is often used in the form of powder (nygrodhadi kvatha churna) which is a herbal formulation. A general description about *Anthocephalus cadamba* has been summarized in Table 2.[3-5] The analytical parameters of plant mentioned in Table 3.[6,7]

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PHYTOCHEMISTRY

Anthocephalus cadamba primarily consist of indole alkaloids, terpenoids, sapogenins, saponins, terpenes, steroids,fats and

Table 1: Vernacular names of *Anthocephalus cadamba*

S.No.	Vernacular names
1	Sanskrit: Kadambah, Vrtta puspa, Priyaka
2	English: Wild chinchona
3	Hindi: Kadamb, Kadam
4	Assam: Roghu, Kadam
5	Tamil: Vellaikkatampu, Arattam, Kadappai
6	Malayalam: Katampu, Attutekka
7	Kannada: Kadamba mara, Kadavala, Neirumavinamara
8	Telugu: Kadambamu, Kadimi chettu
9	Indonesia: Jabon
10	Malaysia: Kalempayan
11	Cambodia: Thkoow

reducing sugars.[1,4] The bark also consist of tannins[2] and an astringent principle; which is due to the presence of an acid similar to cincho-tannic acid.[5] A new pentacyclic triterpenic acid isolated from the stem bark *Anthocephalus cadamba* named cadambagenic acid(18α-olean-12ene-3β-hydroxy 27,28-dioic acid) (Fig. 1), along with this acid quinovic acid (Fig. 2) and β sitosterol (Fig. 3) have also been isolated.[8] Dried stem bark of *Anthocephalus indicus* has been investigated for its steroidal and alkaloidal constituents having good therapeutic values.[9,10] Glycosidic indole alkaloids; cadambine ($C_{27}H_{32}N_2O_{10}$) (Fig. 4), 3α-dihydrocadambine ($C_{27}H_{34}N_2O_{10}$) (Fig. 5), isodihydrocadambine ($C_{37}H_{44}N_2O_{15}$) (Fig. 6)[11,12] and two related non-glycosidic alkaloids; cadamine ($C_{23}H_{23}N_3O_4$) (Fig. 7) and isocadamine isolated from the leaves of *Anthocephalus cadamba*.[13] The isolation and structure of 3β-dihydrocadambine and 3β-isodihydrocadambine (Fig. 8) alkaloids reported from the leaves of *Anthocephalus cadamba* with molecular formula ($C_{37}H_{44}N_{15}O_2$).[14] A new saponin named saponin B ($C_{48}H_{76}O_{17}$) reported from *Anthocephalus cadamba* (Miq.).[15] Anthocephalus cadamba also contain an acid called chlorogenic acid (CGA) (Fig. 9).[16] Recently some worker isolated two novel triterpenoid saponins, phelasin A and phelasin B from the bark of *Anthocephalus cadamba* (Roxb.) Miq.[17] Two novel monoterpenoid indole

Table 2: General description of *Anthocephalus cadamba*

S.No.	Description
1	Habitat: The tree is frequently found all over the India and it is also distributed in Thailand, Indo-china and east-ward in Malaysian archipelago to Papua New Guinea.
2	Parts used: Dried fruits, fresh fruits, leaves, flowers, barks, seeds and roots. Bark: Bark is dark brown, roughish, with longitudinal fissures peeling off in thin scales.
3	Leaves: Leaves coriaceous, elliptical-oblong or ovate, entire margin, pulvinus base, acute shortly acuminate, 18-30 cm long and 10-16 cm broad pubescent beneath.
4	Flowers: Flowers are small, yellow or orange in colour, globose heads which are in 3-5 cm in diameter.
5	Fruits: Fruit a fleshy, orange, globose pseudocarp 5-7 cm in diameter and yellow when ripe.
6	Seeds: Seeds are small, muriculate.

Table 3: Analytical parameters of *Anthocephalus cadamba*

S.No.	Parameters	Result
1	Foreign matters	Not more than (nmt) 2%
2	Total ash	8-9%
3	Acid-insoluble ash	0.6-1.5%
4	Water-soluble ash	2-2.5%
5	Sulfated ash	4-4.5%
6	Alcohol-soluble extractive values	4-6%
7	Water-soluble extractive values	4.5-5.0%

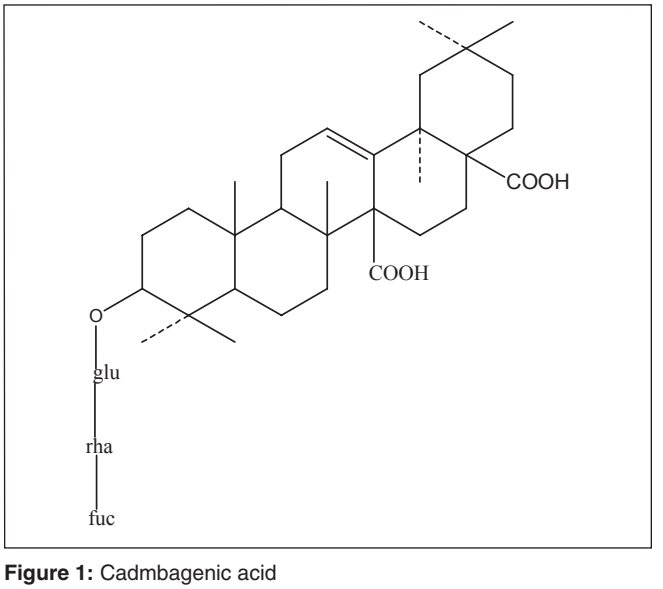

Figure 1: Cadmbagenic acid

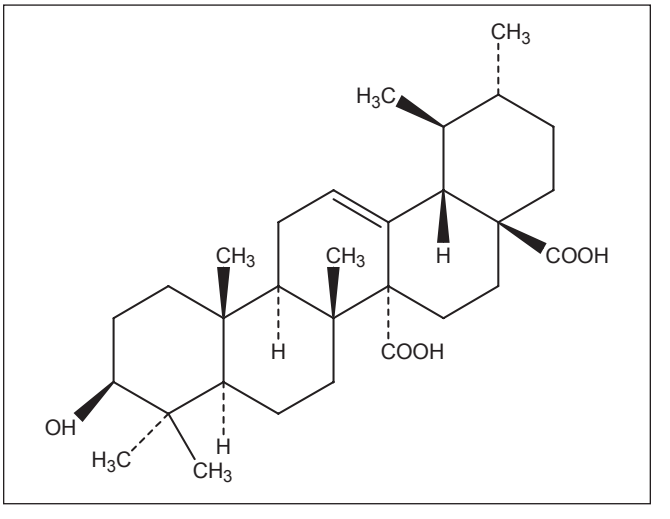

Figure 2: Quinovic acid

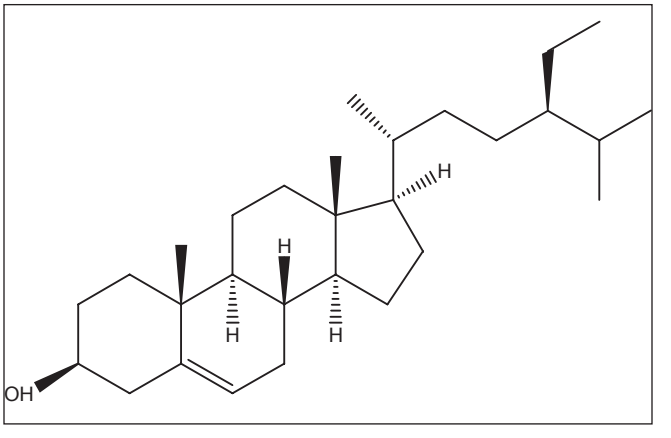

Figure 3: β - sitosterol

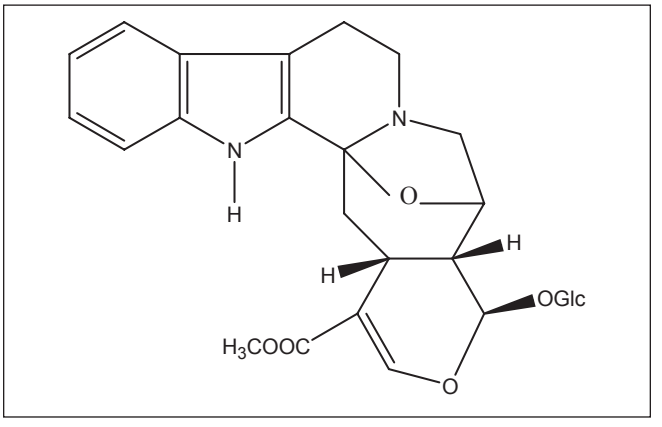

Figure 4: Cadambine

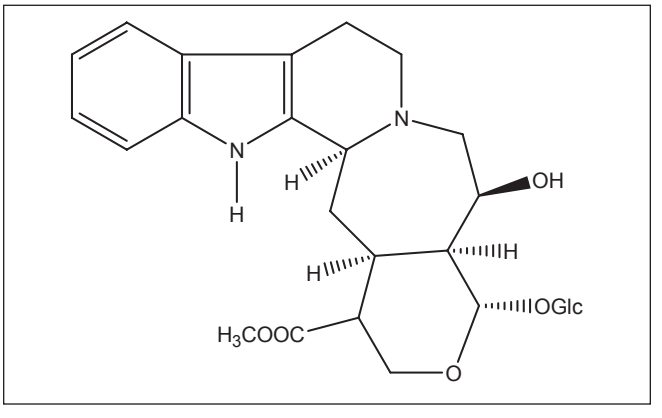

Figure 5: 3α-dihydrocadambine

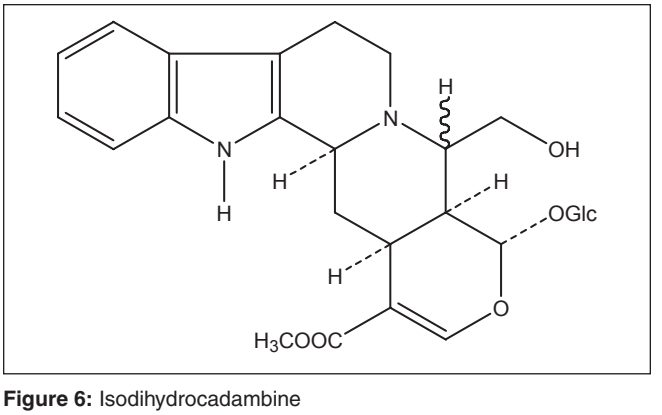

Figure 6: Isodihydrocadambine

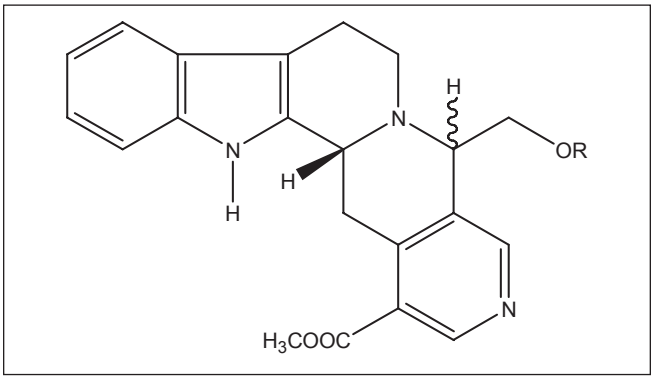

Figure 7: Cadamine

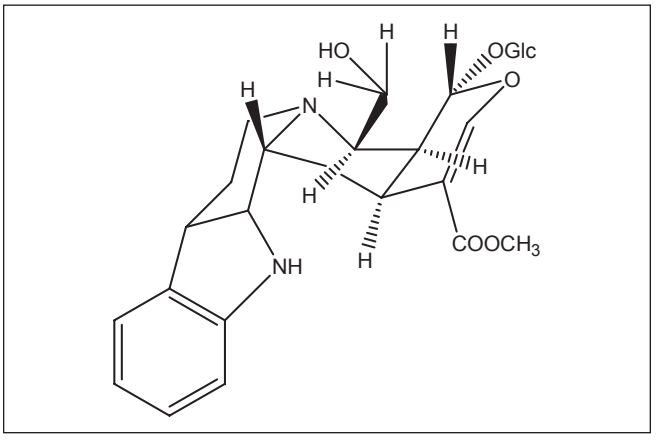

Figure 8: 3β-isodihydrocadambine

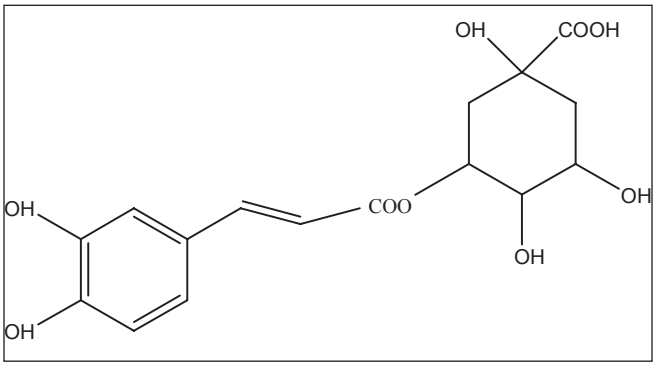

Figure 9: Chlorogenic acid

alkaloids, aminocadambine A ($C_{24}H_{27}N_3O_5$) (Fig. 10) and aminocadambine B ($C_{25}H_{29}N_3O_5$) (Fig. 11) obtained from the leaves of *Neolamarckia cadamba*, previously named *Anthocephalus chinensis*[18] whereas some worker biosynthetically synthesized glucosidic indole alkaloid cadambine from its biological precursor secologanin[19] which is the main precursor of various indole alkaloids. Three monoterpenoid gluco-indole alkaloids, 3β-isodihydrocadambine, cadambine and 3α-dihydrocadambine isolated from *Nauclea cadamba* (Roxb.).[20] The flowers of *Anthocephalus cadamba* yield an

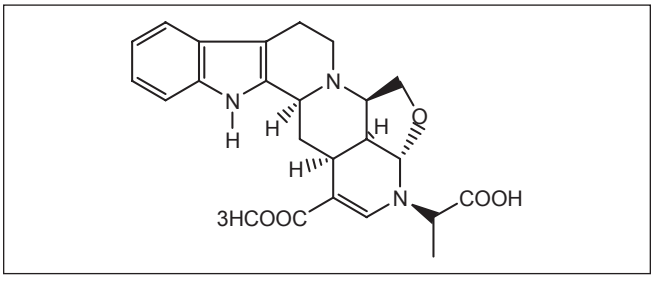

Figure 10: Aminocadambine A

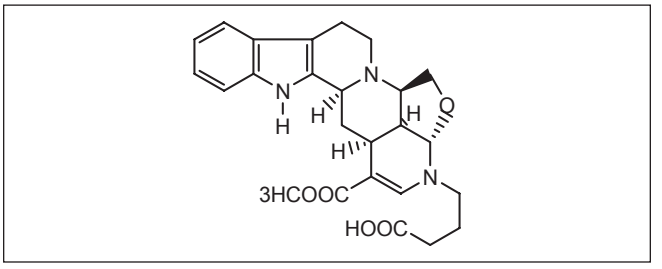

Figure 11: Aminocadambine B

essential oil and the main constituents of oils are linalool, geraniol, geranyl acetate, linalyl acetate, α-selinene, 2-nonanol, β-phellandrene, α-bergamottin, *p*-cymol, curcumene, terpinolene, camphene and myrcene.[2] Two triterpenoid glycosides, glycosides A and B were isolated from the bark of *Anthocephalus cadamba* and defined as 3-*o*-(α-L-rhamnopyranosyl)-quinovic acid-28-*o*-(β-D-glucopyranosyl) ester and 3-*o*-(β-D- glucopyranosyl)-quinovic acid-28-*o*-(β-D- glucopyranosyl) ester respectively[21] and eight different alkaloids also obtained from *Anthocephalus cadamba* named cadambine, CFJ 83, isomalindan, cadamine, 2 derivs. HFP34, GZM28, malindan, dihydrocadambine (Fig. 12), 2 derivs. GPX71, GPX73, isomalindan, isodihydrocadambine, 2 derivs. GPX51, GPX53, malindan.[22] The seeds of *Anthocephalus indicus* composed of water-soluble polysaccharides D-xylose, D-mannose and D-glucose in the molar ratio 1:3:5.[23]

PHARMACOLOGICAL STUDIES

From literature survey it was found that the almost all parts of the plant *Anthocephalus cadamba* is used in the treatment of various diseases. Decoction of leaves are used as gargle in aphthae or stomatitis and in the treatment of ulcers, wounds, and metorrhea. Bark of the plant is used in fever, inflammation, cough, vomiting, diarrhoea, diabetes, burning sensation, diuresis, wounds, ulcers and in the treatment of snake-bite.[1,2,3,5]

Antidiabetic activity

The alcoholic extract of the stem bark of *Anthocephalus cadamba*, syn. *Neolamarckia cadamba* has been reported to exhibit antidiabetic (hypoglycemic) potential in alloxan (120-150 mg/kg) induced diabetic rats and rectifying the problems like fatigue and irritation associated with this disease. The experimental studies showed that the 400-500 mg/kg extract of drug are effective in the treatment of diabetes and it is thought to be due to the presence of flavonoids, which stimulate the insulin secretion or possess an insulin-like effect.[24,25] The alcoholic and aqueous extract of the roots of *Anthocephalus cadamba* also possess the anti-diabetic activity in dose 400 mg/kg body weight and was

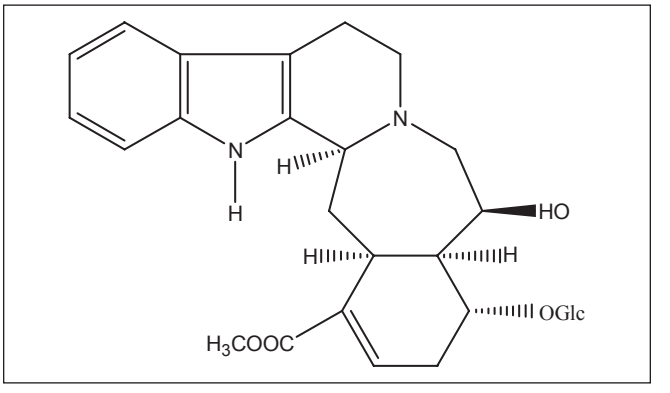

Figure 12: Dihydrocadambine

tested against the normoglycaemic and alloxan induced hyperglycaemic rats.[26]

Analgesic, Antipyretic and Anti-inflammatory activities

Extracts of the bark and leaf of *Anthocephalus cadamba* possess the analgesic, antipyretic and anti-inflammatory activities. The defatted aqueous extract of the leaves of *Anthocephalus cadamba* showed significant analgesic and anti-inflammatory activity at varying doses (50, 100, 300 and 500 mg/kg).[27,28] The methanolic extract of the bark of *Anthocephalus cadamba* was successfully evaluated for analgesic, antipyretic and anti-inflammatory activities by some workers.[29,30]

Antidiarrhoeal activity

The dry hydroethanolic extract (200-500mg/kg) of the flowering tops of *Anthocephalus cadamba* exhibited a dose-dependent decrease in the frequency of faecal dropping in castor oil induced diarrheoea in mice. The extract also produced a dose-dependent reduction in intestinal fluids accumulation.[31]

Diuretic and Laxative activity

The various extracts of the barks of *Neolamarckia cadamba* were studied for its diuretic and laxative activity and it was found that the methanol extract (300 mg/kg) of the bark of *Neolamarckia cadamba* significantly showed in increases the urinary output (diuresis) as compared with aqueous, chloroform and petroleum ether extract, whereas the chloroform extract (300 mg/kg) produced significant laxative property.[32]

Antihepatotoxic effects

Anthocephalus cadamba have been reported to be used for its hepatoprotective activity. The hepatoprotective activity is

due to the presence of chlorogenic acid (CGA) isolated from *Anthocephalus cadamba*. It was also found that the intraperitoneal administration of CGA to mice at a dose of 100 mg/kg for 8 days exhibited a better liver protective action than silymarin (SM), in CCl_4 administered mice. The antioxidative activity of CGA is responsible for its hepatoprotective nature. CCl_4 is used as a model of liver injury.[16]

Hypolipidemic activity

From the experimental studies carried out by the workers showed the marked decrease in the lipid level in alloxan (150 mg/kg body wt.) induced diabetic rats. Oral administration of root extract (500 mg/kg body wt.) of *Anthocephalus indicus* for 30 days in dyslipidemic animals resulted in significant decrease in total cholesterol, phospholipids, triglycerides and lipid peroxides.[33]

Antioxidant activity

The extract of *Anthocephalus cadamba* Syn. *A. indicus* possesses potent antioxidant activity by inhibiting lipid peroxidation and increase in the superoxide dismustase (SOD) and catalase activity.[33,34]

Antimicrobial and wound healing activity

Anthocephalus cadamba has been reported for antimicrobial activitites. The plant have been reported to posses potent antibacterial and antifungal activity against *Escherichia coli, Micrococcus luteus, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumonia, Proteus mirabilis, Candida albicans, Trichophyton rubrum, Asperagillus niger, Asperagillus flavus* and *Asperagillus nidulans*.[34,35] The experimental evidence also show that *A. cadamba* extract has potent wound healing capacity.[34] The aqueous extract of *A.cadamba* also found effective against *Rathyibacter tritici* a causal organism of tundu disease of wheat,[36] and effective against foot and mouth disease of animals.[37]

Anthelmintic activity

Aqueous and ethanolic extracts of mature bark of *Neolamarckia cadamba* has been reported for its anthelmentic activity against earthworms, tapeworms, and roundworms.[38]

Toxicological studies

The methanolic extract of *Neolamarckia cadamba* barks were studied for its toxicity in mouse models. The results suggested that acute toxicity was found in animal models at doses range higher than 3000 mg/kg and there was no mortality found at 3000 mg/kg dose in animal models. The sub-acute toxicity was carried out at dose 600 mg/kg. From the result it is suggested that *N. cadamba* is non-toxic at doses of 600 mg/kg.[29,39]

FORMULATION AND ANALYTICAL STUDIES

Anthocephalus cadamba have been widely used in the in various ayurvedic formulation in the form of churna (nygrodhadi kvatha churn) and oil (grahanimihira taila).[4] It is widely used in the form of paste by tribe in western ghats for treating skin diseases.[34] Earlier various methods have been developed to analyze *Anthocephalus* extract, these method include HPTLC, TLC, and various spectroscopic techniques like IR, Mass and NMR spectroscopy.[40]

CONCLUSION

Research in medicinal plant has gained a renewed focus recently. The main reason is that the other system of medicine associated with number of side effects that often cause to serious problems. Though *Anthocephalus cadamba* has various medicinal activities but it is time to explore its medicinal values at molecular level with the help of various biotechnological techniques. Few toxicological studies have been reported. The work could also be done in this direction to ensure free utility of the plant.

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Withania somnifera (L.) Dunal(Pharmacology Activity)

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ABSTRACT

Withania somnifera has a long medicinal history dating back 5000 years. Ashwagandha or Withania Somnifera is a very significant herbal drug in Unani system of medicine. This meticulous herb was used to treat a variety of infectious diseases as well as tremors and inflammation especially osteoarthritis, rheumatoid arthritis, and gout. Ashwagandha or Withania Somnifera is a stunning delicate plant inhabitant to Indian subcontinent. Defined by green leaves, branched limbs, and topped by seeded yellow flowers. Ashwagandha or Withania Somnifera is an eye-catching in look and of very importance in Ayurveda or Indian System of Medication. Ashwagandha or Withania Somnifera has a vast history, about 5000 years, as a holistic herb used to treat a variety of ailments.

BOTANICAL AND PHYSICAL CHARACTERISTICS

An evergreen shrub growing to 1 m to 0.5 m. It is in leaf all the year. The flowers are hermaphrodite (have both male and female organs). The plant prefers light (sandy), medium (loamy), and heavy (clay) soils and requires well drained soil. It can not grow in shade. It requires moist soil.

Active constituents: withaferin A and withanolide D. Anaferine (alkaloid), anahygrine (alkaloid), beta-sisterol, chlorogenic acid (in leaf only), cysteine (in fruit), cuscohygrine (alkaloid), iron, pseudotropine (alkaloid), scopoletin, somniferinine (alkaloid), somniferiene (alkaloid), tropanol (alkaloid), withanine (alkaloid), withananine (alkaloid) and withanolides A-Y(steroidal lactones), somniferine, somnine, pseudo-withanine, tropine, pseudo-tropine, 3-a-gloyloxytropane, cuscohygrine, isopelletierine, anahydrine, sitoindoside VII and sitoindoside VIII, pseudo-withanine, tropino, choline, cuscohygrine, isolettetierine, anahydrine, 3-alpha-gloyloxy tropane[1]

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MEDICINAL USES AND PHARMACOGICAL ACITIVITY

Withania somnifera has been used in Unani Medicine as an anti-inflammatory and to treat rheumatism beside other ailments. Withania somnifera is recommended as having Abortifacient; Anti-inflammatory, Adaptogen; Antibiotic; Aphrodisiac; Astringent; Deobstruent; Diuretic; Sedative; Tonic. According to Unani system of medicine, the root is bitter, tonic, aphrodisiac, emmenagogue, anti-inflammatory. Studies have shown asgandh to be effective in stimulating the immune system. It also appears to inhibit swelling and aid memory It is especially beneficial in stress related disorders such as arthritis and premature aging. Withanolides possess remarkable antitumour, antiarthritic, anti-inflammatory and immunosupressive properties. Asgandh is used for treatment of rheumatism, hyperuricemia, and as sex stimulant. Asgandh is one of the most widespread tranquillisers used in India and Pakistan, where it holds a position of importance similar to ginseng in China and Korea. It is an anti-inflammatory agent. It is used to improve vitality and aid recovery after chronic illness. The leaves and the root bark, are deobstruent, diuretic, narcotic, strongly sedative and tonic. It is also used to treat, debility, insomnia, impotence, infertility, multiple sclerosis etc. The seed is diuretic and hypnotic. Withania Somnifera has a long history, of about 4000 years, as a holistic herb used for treating a variety of ailments. This particular herb is used to treat various infectious diseases as well as tremors and inflammation especially arthritis, nervousism, insomnia, weakness, anemia, rheumatic pains, general debility among other conditions.

It's a boon in cases of impotence and infertility and is very useful in spermatorrhea, loss of strength, lack of sexual drive, erectile dysfunction. It is a good Uterine anti-inflammatory so useful in Uterine infections. Thus it is considered to be the best aphrodisiac drug. Ashwagandha stimulate the immune system, stop inflammation, increase memory and helps maintain general health and wellness of the body. It is high in iron content and increases hemoglobin. The fruits of Ashwagandha are a blood tonifier, so when consumed over a considerable period of time improves circulation of blood in the body and absorption of nutrients by cells. Also it is known to increase the production of bone marrow, semen and inhibiting the ageing process. All anxiety symptoms including panic attacks and paranoia had virtually disappeared by the use of this drug. If taken in proper dose, it can restore the neurotransmitters and hence have found to be useful in treating various mental disorders like Depression, Alcohol dependency and Schizophrenia. Also, by taking Ashwagandha regularly one can enhance his memory and learning skills dramatically. Many studies have been conducted to see the effects of Ashwagandha on tumors, arthritis and other forms of inflammation. The studies proved that Ashwagandha acts as an anti-tumor, anti-inflammatory agent. It's naturally occurring steroidal content is much higher than that of hydrocortisone which is a common treatment in cancer cases[2-4].

DIABETES

The hypoglycemic, diuretic and hypocholesterolemic effects of roots of Ashwagandha were assessed in six patients with mild NIDDM and six patients with mild hypercholesterolemia. The treatment consisted of the powder of roots over a 30 day period. At the end of the study, researchers noted a decrease in blood glucose comparable to that of an oral hypoglycemic drug, and a significant increase in urine sodium and urine volume, coupled with a decrease in serum cholesterol, triglycerides, LDL (low density lipoproteins) and VLDL (very low density lipoproteins) cholesterol, with no adverse effects noted[5,6]

IMMUNITY

Myelosuppressed mice treated with an extract of Ashwagandha displayed a significant increase in hemoglobin concentration, red blood cell count, white blood cell count, platelet count and body weight as compared to controls, as well as increased hemolytic antibody responses towards human erythrocytes[7]. Researchers at the Amala Cancer Research Centre in Kerala, India, found that the administration of an extract from the powdered root of Withania somnifera enhanced the levels of interferon, interleukin-2 and granulocyte macrophage colony stimulating factor in normal and cyclophosphamide-treated mice, suggesting an immunopotentiating and myeloprotective effect. Mice infected intravenously with Aspergillus fumigatus and treated for 7 consecutive days with an oral preparation of an extract of Withania somnifera at a dose of 100mg/kg displayed increased phagocytic activity and prolonged survival time[8-9] The antifungal activity of Withania has been confirmed elsewhere, attributed to the withanolides[10].

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