Pharmacognostical Investigation of *Ionidium suffruticsoum* Ging. – A Seasonal Multipotent Medicinal Herb.

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ABSTRACT

Ionidium suffruticosum Ging. is a seasonal multipotent medicinal herb belongs to the family Violaceae. The plant claims highest medicinal values and used traditionally to treat diabetes, jaundice, male sterility, urinary tract infections, gonorrhea, bowel complaints, urinary problems and various ailments. No detailed pharmacognistical works are available, hence, in the present study, morphological, microscopical and preliminary phytochemical investigations of the herb were undertaken. All the parameters were studied according to the WHO & Pharmacopoeal guidelines. Anatomical studies showed that secondary growth occurs in roots and basal stocks of stem indicating the seasonal and perennial nature of the herb. Powder microscopy revealed that phloem fibres, xylem vessels with spiral, annular, scalariform lignifications and calcium oxalate crystals were abundant in dried material of the plant. The qualitative chemical tests of the petroleum ether, chloroform, methanol and aqueous extracts of plant material revealed the presence of alkaloids, steroids, triterpenoids, flavonoids and carbohydrates.

Keywords: *Pharmacognosy, Ionidium suffruticosum, Phytochemical screening, Violaceae.*

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INTRODUCTION

Ionidium suffruticsoum, Ging. Syn. *Hybanthus enneaspermus* (L). F. Muell. belongs to the family Violaceae, is a rare ethnomedicinal ephemeral herb[1] or under shrub (Resembling a shrub, especially in having basal woody stems and branches (Latin fruticosus means bushy stem). Plants are distributed in the tropical and subtropical regions of the world. It is an herb, found in the warmer parts of India. The plant is popularly called as Spade Flower (English), Purusharathna (Kannada) and Ratan purush (Hindi). Spade Flower is a perennial herb or small shrub of 40 to 60 cm height with a long slender tap root. The leaves are sub sessile, linear, lanceolate, margin serrate, apex acute and stipules acuminate, 1–4 mm long. Pink-purple spade-shaped flowers solitary. Sepals 3–4 mm long. Lower petal broad spade-shaped, pink-purple, with deep purple veins. Upper petals linear-oblong, 3–4 mm long; lateral pair 4.5–5 mm long. The fruits are capsules 4–9 mm long; ribbed seeds 5–12, pitted between ribs. Flowering is from June to November in India. Plants are found along river banks, open grasslands, sandy places and rocky regions. In nature (*in vivo*) the plants are seasonal and appear for few months. The roots and few basal stem stocks retaining in the soil and are regenerating during rainy season and soon after the rainy season the aerial part dries up and the plants disappear.

The plant is considered to have highest medicinal value and widely used by traditional healers to treat several diseases like diabetes,[2] malaria (antiplasmodial activities[3]), male sterility in Ivory Coast,[4] urinary tract infections and water retention and is used as tonic.[5] The tender leaf stalks are used as demulcent; the roots are antigonorrhoeic, diuretic, bowel complaints and urinary problems.[1] Despite its multipotentiality as a medicine there are some limitations in the propagation of this species. Propagation of the species by seeds or any other conventional methods are not reliable because the plants are seasonal and available for short duration in nature. Moreover, the seeds are viable for short period and loose their viability within few weeks. The species are under threat due to their exploitation from their natural habitat

by traditional healers, over grazing by animals, seasonal habitat and their short seed dormancy. At the same time, no data are available concerning its biological activities[6,7]. The present communication reports the macroscopic, microscopic, dry powder analysis in different solvents/ reagents facilitates the correct identification of the dried plants or powdered drug which detects and prevents the adulteration(s), if any. The challenge ahead of this is to authenticate the therapeutic efficacy and safety of the plant, using standard methods.

MATERIALS AND METHODS

The studies were executed in the fresh specimens of *Ionidium suffruticsoum* collected from their natural habitat, local area of Hubli and were authenticated by one of the authors, Dr. M. Jayaraj. A voucher specimen has been deposited in the P. G. Department of Botany, Karnatak University, Dharwad for future reference. The external morphology of plant parts and other structural peculiarities were studies in the macroscopic observation. Anatomical characters of root, stem, leaf and stomatal characters were included in the microscopic investigations. The dried material of the plant was stored under normal environmental conditions. The Physico-chemical parameters such as extractive values, ash values, loss on drying were performed as per the official standard procedures.[12,13] Microscopical investigations were made by microtome sections and powder microscopy was performed according to the prescribed procedure.[14,15]

The fresh parts of the plant are subjected to morphological characterization. Fresh hand cut sections of the root, stem and leaf were taken from the fresh material as per method described by Trease and Evans.[8] T. S. of root, stem and leaf were stained with saffranin and Hematoxylin. The microphotographs were taken by trinocular microscope with digital Olympus Camera for detailed studies.

The plant material collected from their natural habitat was cleaned, shade dried at room temperature, coarsely powdered and stored in an air tight glass container. 100g of coarse powder was successively extracted with petroleum ether (40–60), chloroform and methanol in Soxhlet extractor for 18 hours. The extracts were filtered and concentrated using rotary flash evaporator and residues were dried in desiccators over sodium sulfite below 60°C. Freshly prepared extract was subjected to phytochemical evaluation for the detection of various constituents using conventional protocol.[9]

Physico-chemical parameters were determined as per Indian Herbal Pharmacopoeia and were analysed for moisture content, total ash, water sulphated ash, petroleum ether soluble extract, water soluble ash, alcohol soluble extract and water soluble extract. Preliminary phytochemical investigations of whole plant extract was carried out to know the medicinally important chemical constituents. The methanol, petroleum ether, chloroform and aqueous extracts of the plant were subjected to the different chemical tests to find out the nature of chemical constituents.

RESULTS AND DISCUSSION

Pharmacognistical investigations:

The detailed and systematic pharmacognistical evaluation would give valuable information for the future studies. The detailed morphology of *Ionidium suffruticosum* Ging. was carried out to support proper identification of drug . Stomata and stomatal index: The stomata are predominantly paracytic (Rubiaceous or parallel celled) and some are anisocytic (Cruciferous or unequal celled). The epidermal cells are larger than subsidiary cells. Stomatal index, the percentage of stomata found in unit area of leaf exhibited marked variation in the adaxial and abaxial surface of the leaf. Abaxial surface has an increased stomatal frequency than adaxial surface (Fig. 1 D and (E). The values are represented in the Table 1.

Organoleptic, anatomical and powder microscopic study

The powder of *I. suffruticosum* is dull brown. It has no characteristic odour or taste. Microscopic examination of the powdered plant material indicated the presence of xylem vessels which are lignified, bordered pitted, spiral, annular and scalariform thickenings of varying length were associated with fibres in bundles. Apexes of the fibres were bluntly pointed. Thick walled xylem fibres and phloem fibres were present in large number. There are unbranched unicellular trichomes or hairs of specific form and structures and function were noted. Powder microscopy showed the presence of thick and thin walled cork cells, cortex cells containing calcium oxalate crystals and medullary rays crossing across the cortex.

The T. S of fresh part of the root showed the presence of bark, secondary cortex, annular rings, secondary medullary rays and compressed xylem at centre indicate the roots had secondary growth and perennial in nature. The T. S. of fresh stem revealed the presence of thick cuticle, compact layer of epidermis, trichomes, distinguished cortex, endodermis, semilunar sclerenchyma patches, a cambium tissue separating phloem outside and wider

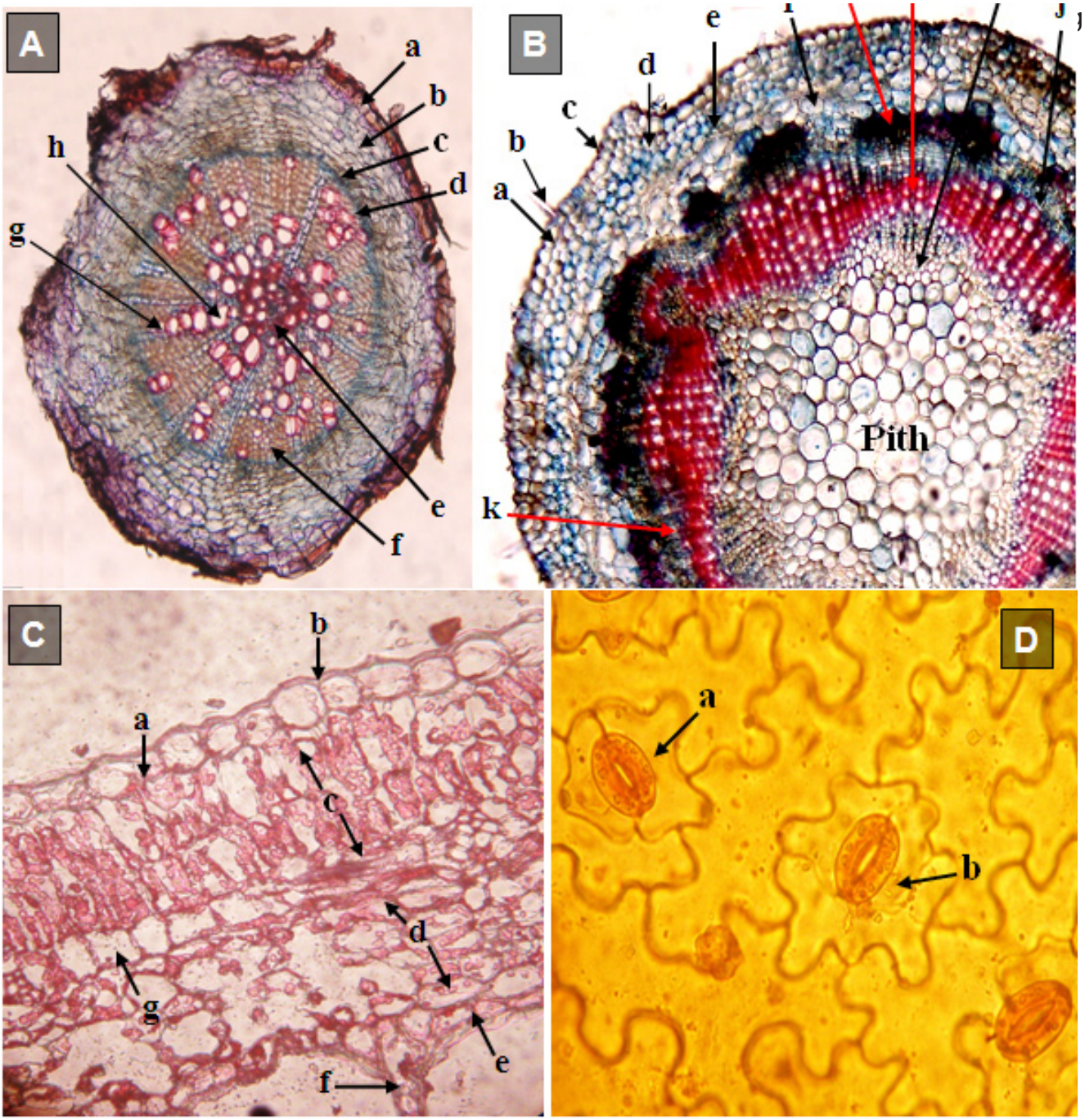

Figure 1. A – T.S. of young root a-epidermis, b-cortex, c-endodermis, d-pericycle, e-medulla filled with xylem tissue, f-phloem, g-protoxylem, h-metaxylem. B- T.S. of stem, a-epidermis, b-stem hair, c-cuticle, d-hypodermis, e-cortex, f-endodermis, g- sclerenchyma patches, h-secondary xylem, i-primary xylem, j-secondary phloem, k-vascular cambium. C – T.S. of leaf, a-upper epidermis, b-cuticle, c-palisade parenchyma, d- spongy parenchyma, e- lower epidermis, f- leaf hair, g-separating layer between palisade and spongy parenchyma. D- Surface view of epidermis showing a-paracytic stomata and b- anisocytic stomata.

secondary xylem and primary narrow xylem cells towards inside. Abundant pith (parenchyma) encloses the centre. The T.S. of fresh leaf showed the presence of the upper and lower epidermis enclosing the compact mesophyll. A distinct separating layer between the palisade and spongy parenchyma is a peculiar character.

Physicochemical and Preliminary Phytochemical Studies

Physical constant values like extract values, ash values and moisture content are tabulated in Table 1. Preliminary phytochemical tests of plant part extracts were conducted

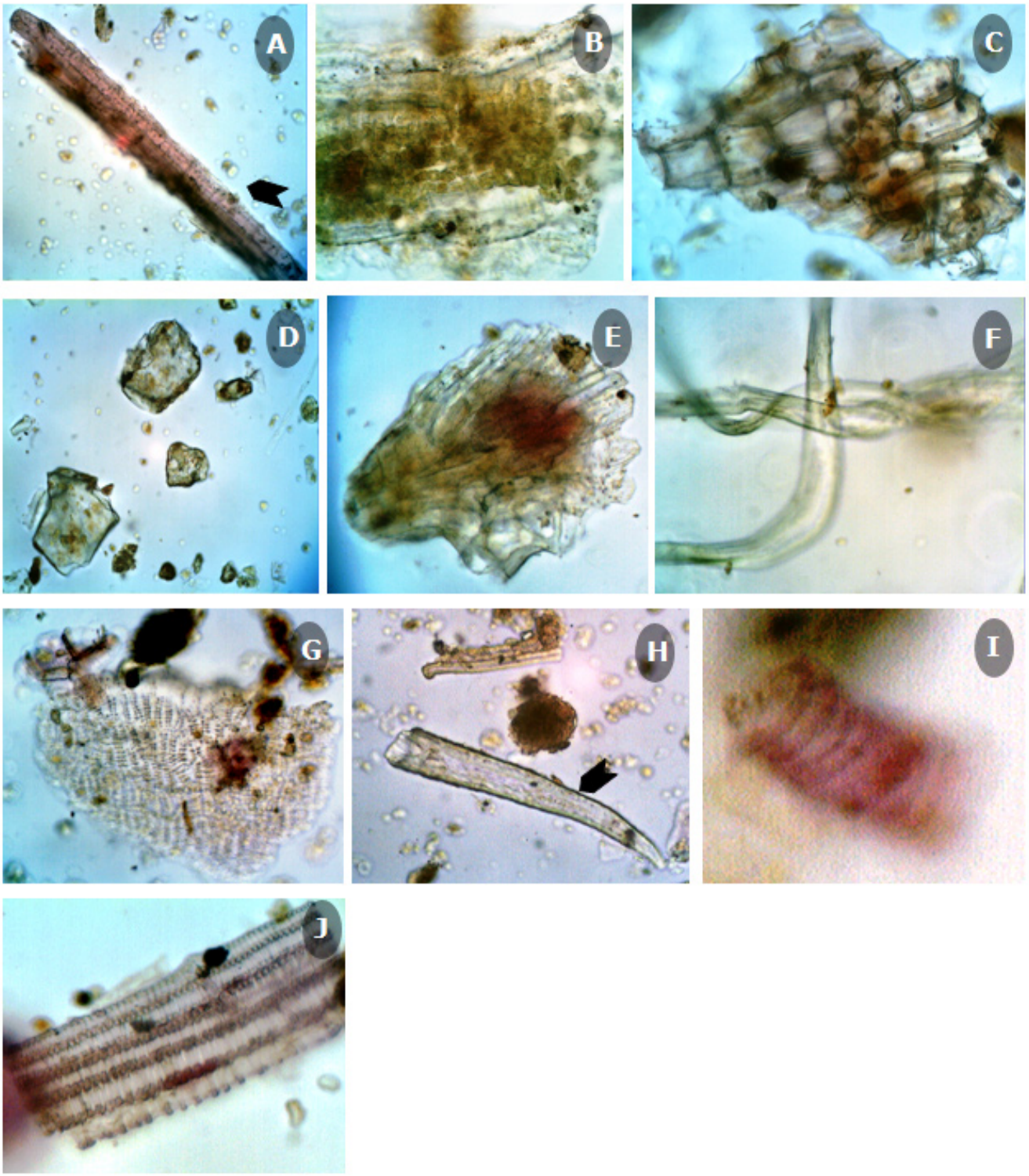

Figure 2. A-phloem fibres B-Part of lamina with upper palisade and lower spongy parenchyma C-cortex of stem D-crystal forms of calcium oxalate E-testa F-medullary rays G-medullary along with sieve tissue H-covering trichomes I spiral thickenings of vessel J-annular, spiral, reticulate & bordered pitted vessels.

separately and detected for different medicinally important chemical constituents. The alcoholic extracts of the different organs of the plant i.e., roots, leaves, flowers, fruits and seeds were subjected to the different following chemical tests and found out the nature of constituents present in the plant organs.

Alcoholic extracts showed that roots, leaves, fruits and seeds contain steroids. Triterpenoids and flavanoids

Table 1. Stomatal index on adaxial and abaxial leaf surfaces of *Ionidium suffruticsoum*.

Trials	Adaxial surface of leaf		Abaxial surface of leaf	
	Margin	Middle	Margin	Middle
1.	167	143	153	218
2.	186	156	163	234
3.	179	148	158	212
4.	168	145	172	225
5.	176	151	176	234
6.	183	153	160	229
Average	176.5	149.34	163.67	225.34
	162.92		**194.50**	

are present only in flower. Alkaloids are present in roots, leaves and flowers except fruits and seeds. Saponins, Proteins Glycosides, amino acids and Tannins are absent in plant. Carbohydrates are present in roots, leaves, fruits and seeds except flowers. Only the seeds contain fatty acids.

Conclusions

Pharmacognistical analysis of I. suffruticosum showed many important features useful for the identification of the drug plant. Macroscopic characters like secondary growth in root and basal stems depicting the name suffruticosum bearing bushy nature although it is a herb. Presence of single row of sub epidermal collenchyma and chlorenchymatous cortex are well indicating the herbaceous character of the plant. Massive schlerenchymatous patches, abundant xylem vessels and phloem fibres are unique to plant. Hypostomatic leaves, flat upper surface and semicircular lower surface of the midrib were the important diagnostic feature of the drug plant. Predominantly paracytic and some anisocytic type of stomata are useful identification marks of the plant. High stomatal frequency establishes the xerophytic and ephemeral character of *I. suffruticosum*. Morphological and anatomical studies revealed that the plant is seasonal and perennial herb available for short period to traditional healers and for other preparation. Due its high medicinal values the plant is continuous exploitation is in alarming rate. The presence of alkaloids, steroids, Triterpenoids, Flavanoids and carbohydrates (Table No. 2) suggests that *Ionidium suffruticosum* have valuable and medicinally important chemical ingredients in its different organs. It is present need to conserve the plant for medicinal usage. Tissue culture techniques may be more useful in the conservation point of view.

Table 2. A. Extractive values B. Moisture content and C. Ash values of *Ionidium suffruticosum* (whole plant).

Parameter	Determined value (%)
Extractive values	
Alcohol soluble extractive value	5.60
Water soluble extractive value	11.88
Ether soluble extractive value	8.0
Moisture Content	8.74
Ash Values	
Total ash	18.50
Water soluble ash	2.97
Sulfated ash	23.50

Table 3. Qualitative chemical analysis of various extracts of *I. suffruticosum* Ging.

Tests	PE	$CHCl_3$	MeOH	AQ
Alkaloids	–	+	+	–
Glycosides	–	–	–	–
Steroids	+	–	+	+
Triterpenoids	–	+	–	–
Saponins	–	–	–	–
Flavanoids	–	+	+	–
Tannins	–	–	–	–
Carbohydrates	+	–	+	+
Amino acids	–	–	–	–
Fatty acids	+	–	–	–

Note: PE = Petroleum Ether, $CHCl_3$ = Chloroform, MeOH = Methanol, AQ = Aqueous; + indicate presence – indicates absence

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Table 4. Qualitative chemical analysis of alcoholic extracts of different parts of *I. suffruticosum*, Ging.

Tests	Roots	Leaves	Flower	Fruits	Seeds
Alkaloids	+	+	+	–	–
Glycosides	–	–	–	–	–
Steroids	+	+	–	+	+
Triterpenoids	–	–	+	–	–
Saponins	–	–	–	–	–
Flavanoids	–	–	+	–	–
Tannins	–	–	–	–	–
Carbohydrates	+	+	–	+	+
Amino acids	–	–	–	–	–
Fatty acids	–	–	–	–	+

Note: + = Present – = Absent.

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Pharmacognostical, Phytochemical and Anthelmintic Evaluation of *Leucas indica* (L)

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ABSTRACT

The present study deals with pharmacognostical, phytochemical and anthelmintic evaluation of entire plant of *Leucas indica*. This evaluation reveals the presence of many phytochemical constituents. Entire plant was extracted with petroleum ether, chloroform and methanol. Crude tannins were isolated from methanol extract and evaluated for anthelmintic activity. Chloroform methanol and crude tannin extracts showed very good activity. Paralysis and death time of crude tannins were very close to standard drug Albendazole.

Keywords: *Leucas indica*, crude tannins and anthelmintic activity..
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INTRODUCTION

*Leucas indica (*Linn) belonging to the family of Lamiaceae is commonly known as Tummi in Telugu. *Leucas indica* is distributed throughout India in common along road ways, waste lands, and river banks and on rocky hills. *Leucas indica* is an erect herb, branches are appressed and pubescent. Leaves are linear-lanceolate, entire undulate or distantly serrate. Flowers are white, shortly pedicelled in whorls, towards the end of the branches. Calyx tube slightly curved, 8- toothed, posterior tooth longer than the rest. Corolla is annulated within near the middle. Stamens are 4. Leaves are used as vermifuge, stomachic, sedative and sores.[1] Phenylethanoid glycosides were isolated from the aerial parts of *Leucas indica Linn* and they were found to contain antioxidant activity along with the inhibitory activity against xanthine oxidsase enzyme.[2] Methanolic extract of aerial parts of *Leucas indica* showed antipyretic activity.[3] Methanolic extract was found to show a potential reduction in spontaneous activity and cause a significant decrease in exploratory behavioural pattern by the head dip and Y-maze test. It also shows a significant reduction in muscle relaxant activity by rotarod, 30 inclined screen and traction tests. The extract shows a remarkable potentiation of pentobarbitone induced sleeping time in mice.[4] Methanolic extract of herb caused a significant reduction of blood glucose levels in streptozocin induced diabetes[5] and has antitussive activity [6] as well as wound healing activity.[7]

MATERIALS AND METHODS

Plant material

The whole plant of *Leucas Indica* was collected in February 2008 from Thirumala hills, Andhra Pradesh,India. *Leucas Indica* (L) R .Br. ex Vatke (Lamiaceae) was authenticated by Dr. K. Madhava Chetty, Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. The entire plant was cleaned, dried at room temperature and stored properly in air tight container. The dried plant material was then subjected to size reduction to obtain coarse powder using grinder. This powdered material of no 16 mesh size was then used for further process.

Macroscopic and microscopic analysis

The macroscopy and microscopy of the powder were studied according to the method of Khandelwal and Kokate. For the microscopic studies, cross sections were

prepared and stained as per the procedure prescribed by Khandelwal and Kokate.[8–10] Micro powder analysis was done as per method prescribed by Vijayakumari. [11]

Physico- chemical analysis

Physico-chemical analysis like moisture content, total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive and water soluble extractive values were performed according to the official methods prescribed by Indian Pharmacopeia[12], Khandelwal and Kokate.[8–10]

Preparation of different plant extracts

The powdered plant material (2 kg) was extracted with solvents of increased polarity such as, petroleum ether, chloroform and methanol for 24 h with each solvent by hot extraction using Soxhlet apparatus at 60 °C. The extracts were concentrated under reduced pressure using a rotary evaporator to constant weight. The extracts were collected and preserved in a dessicator until used for further studies.

Isolation of crude tannins

1 g of methanolic extract of plant was agitated vigorously with 80% acetone at 55°C in a reactor fitted with propeller, stirrer and proper baffles to produce uniform mixing. The slurry after cooling was filtered and acetone was recovered. The left over slurry was suspended in distilled water (three times) and stirred to form a homogenous solution. It was filtered and dried.[13]

Percentage purity of isolated crude tannins

100 mg of isolated crude tannins were dissolved in 5 ml of water and filtered. From the filtrate 1 ml was pipetted out and diluted to 7.5 ml with water. To this 0.1 ml indigo sulphonic acid solution was added and titrated against 0.01N potassium permanganate solution until the colour changes to golden yellow. Percentage of total tannins was calculated as per standard procedure.[13]

Phytochemical study

Powder of entire plant material was subjected to fluorescence analysis. A portion of residue from each extract and crude tannins from methanol extract was subjected to phytochemical analysis in order to see the presence of steroids, alkaloids, tannins, proteins, glycosides, carbohydrates, phenols, flavonoids, volatile oils, saponins and starch.[8,10]

Thin layer chromatography study

All the extracts of plant were subjected to thin layer chromatographic study to determine the number of spots and corresponding R_f values by developing different solvent systems. TLC was performed using pre-coated silica gel TLC plates of E-Merck, Germany. The developed TLC plates were observed under daylight, UV light, iodine chamber and by spraying various detecting reagents.

Anthelmintic activity

Prepared extracts and isolated crude tannins were evaluated for anthelmintic activity separately. Adult Indian earth worms, *Pheretima posthuma* were chosen for the study due to their anatomical and physiological resemblance with the intestinal round worm parasite of human beings.[14] They were collected from in Nalgonda region and identified by Sri Prasad Traders, Nalgonda, and Andhra Pradesh, India.

The earthworms of nearly equal size (6 cm±1) were acclimatized to the laboratory condition before experimentation. The earth worms were divided into six groups of six earth worms in each. Albendazole was diluted with 5% DMF in Normal saline solution to obtain 10 mg, 25 mg and 50 mg served as standard and poured into petridishes. The extracts were dissolved 5% DMF in normal saline solution and diluted to prepare three concentrations such as 10 mg, 25 mg, and 50 mg and poured into Petri dishes. 5% DMF in normal saline solution was taken as control. Earth worms were placed in Petri dishes containing different concentrations of standard and extracts as well as crude tannins at room temperature. The mean paralysis time and mean death time for each sample was calculated (all the readings were taken in triplicate).The time taken for worms to become motionless was noted as paralysis time and to ascertain death, each worm was frequently applied with external stimuli which stimulates and induce movement in earth worm if alive.[15]

RESUTS AND DISCUSSION

Macroscopic characters of powder – The colour of the powder is light reddish green, odourless and has bitter taste.

Microscopy characters

Transverse section of stem – The stem in cross – sectional view (Fig.1) consists of epidermis. Epidermis was the outer most layer composed of rectangular cells, which were closely arranged without intercellular spaces. Cortex was very small, with the presence of hypodermis. Hypodermis

was very thick found beneath the epidermis where the cell were sclerenchymatous and were arranged compactly without any intercellular spaces in between. Remaining part of the stem was occupied by the ground tissue called parenchymatous tissue with chloroplast. Vascular bundles were present in ground tissue where xylem vessels were separated by the multi seriate medullar rays. Lignified pericyclic fibres were present in form continuous circle. Pith was present in small central parenchymatous portion.

Transverse section of root - Transverse section of root was shown in Fig. 2. Cork appeared as yellowish brown present in many layers. It was followed by cortex which was not so distinct, but the endodermis consisted of rectangular cells which were arranged in single layer. It was followed by 3–4 layers of pericyclic fibres, xylem vessels and phloem cells. The remaining place in between the xylem and phloem is occupied by the parenchymatous cells and pith was present in the form of small central parenchymatous portion.

Powder characters – On microscopial examination the powder showed the following cells (Fig. 3, 4, 5 and 6). Cork cells were thick walled without intercellular spaces. Lignified fibers were thick walled uniform in thickness, long, vertical and present in single fragments. Epidermal cells were thick walled in nature. Trichomes were unicellular head and uniserate in nature. Lignified reticulated xylem vessels were present. The length and width of phloem fibre was found to be 81–124 µm and 30–60 µm respectively. The size of the calcium oxalate was found to be 5–25 µm (length) and 25 µm (width).

Micropowder analysis

Fluorescence analysis of powder – The powdered drug showed color change under visible light and ultraviolet light after treatment with different chemical reagents. This

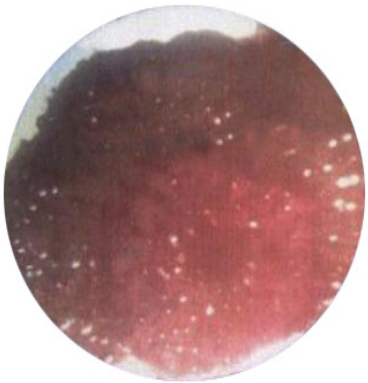

Figure 1. Transverse section of stem of *Leucas indica*

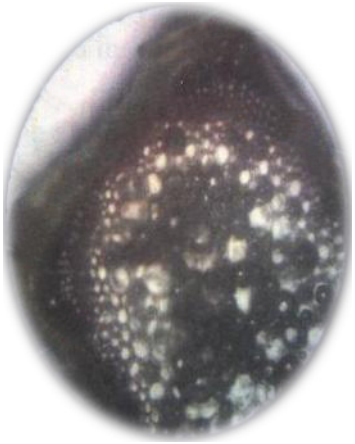

Figure 2. Transverse section of root of *Leucas indica*

Figure 3. Xylem fibres of *Leucas indica*

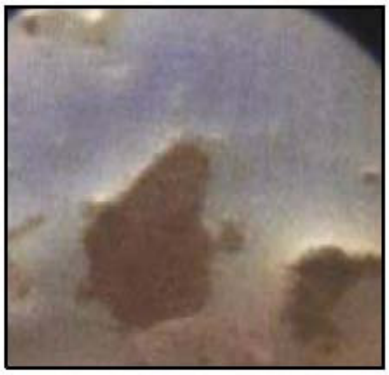

Figure 4. Cork cells of *Leucas indica*

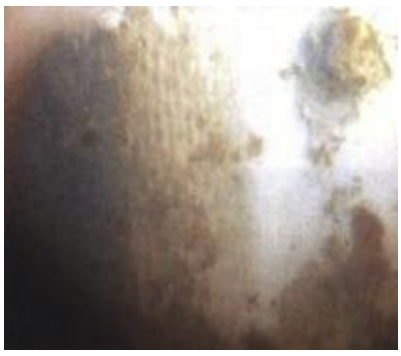

Figure 5. Epidermal cells of *Leucas indica*

Figure 6. Fibres of *Leucas indica*

fluorescence analysis revealed the presence of chemical constituents with fluorescent character. The results were shown in Table 1

Chemical analysis of powder – Treatment of powdered drug with different chemical reagents was revealed the presence of different chemical constituents (Table 2). In this analysis, on treatment with water it produced foam which indicates the presence of saponins. The foaming index was found to be 5%.

Physico-chemical analysis –Acid insoluble ash is used to know the percentage of dirt and sand. Extractive values are primarily useful for the determination of exhausted or adulterated drugs. The ash value, extractive value and loss on drying were shown in Table 3. Total ash values of a drug give information about inorganic compounds such as carbonates, phosphates, silica and silicates, which are naturally occurring in drug or deliberately added to it as a form of adulterant.

Extraction

Powder of the *Leucas indica* was extracted with petroleum ether, chloroform and methanol. % yield of petroleum ether, chloroform and methanol extracts were found to be 2.43 (green color mass), 2.87 (green color mass) and 4.53 (reddish brown color mass) respectively. % purity of tannins in chloroform and methanol extracts were found to be 0.089 ± 0.0577 and 0.16 ± 0.0034 respectively (n=3). Amount of tannin in methanolic extract is greater than chloroform extracts. Therefore, crude tannins were isolated from methanolic extract which yielded 0.0039%.

Table 2: Chemical analysis of entire plant of Leucas indica

Treatment	Observation
Powder as such	Light reddish green
Powder + 2% Ferric chloride	Dark green
Powder + 10% Sodium hydroxide	Yellow green
Powder + Sodium hydroxide +Water	Pale green
Powder + 5% Potassium hydroxide	Brownish green
Powder + Water, shake	Foam formation
Powder + Ethanol	Pale green
Powder + Sulphuric acid	Blackish brown
Powder + Hydrochloric acid	Greenish brown
Powder + Nitric acid	Brown

Table 3: Ash and extractive values of entire plant powder of Leucas indica

Parameters	*Leucas indica*
Total ash	5%
Acid insoluble ash	4%
Water soluble ash	6.8%
Alcohol soluble extractive	10.4%
Water soluble extractive	15%
Loss on drying	3.90%

The % purity of tannins in crude tannins was found to be 0.212 ± 0.0043 (n=3).

Phytochemical analysis

Preliminary Phytochemical screening revealed the presence of various phytochemicals given in Table 4. Petroleum ether extract showed the presence of only carbohydrates. Alkaloids, tannins, glycosides, phenols and volatile oil were found in chloroform extract. Alkaloids, tannins, glycosides, carbohydrates, phenols, flavonoids, and saponin were found in methanolic extract. Crude tannins isolated from methanol were subjected to phytochemical analysis showed positive result for tannins, phenols and flavonoids but negative for Alkaloids, glycosides, carbohydrates and saponins

Table 1: Fluorescence analysis of the entire plant of Leucas indica

Treatment	Visible light	Ultraviolet light
Powder as such	Dark green	Light reddish green
Powder + 5% Sulphuric acid	Reddish brown	Black
Powder + Ethanol	Light green	Dark green
powder + 1N sodium hydroxide	Reddish green	Pale green

Table 4: Phytochemical screening of the entire plant powder of Leucas indica

Constituents	Petroleum ether extract	Chloroform extract	Methanolic extract
Steroids	–	–	–
Alkaloids	–	+	+
Tannins	–	+	+
Proteins	–	–	–
Glycosides	–	+	+
Carbohydrates	+	–	+
Phenols	–	+	+
Flavonoids	–	–	+
Volatile oil	–	+	–
Saponins	–	–	+
Starch	–	–	–

"+" indicates the presence of constituents "–" indicates the absence of constituents

Table 5: TLC analysis of entire plant powder of Leucas indica

Extracts	Solvent system	R_f values
Petroleum ether	Benzene : Diethyl ether [6:1]	0.28, 0.55, 0.75, 0.86.
Chloroform	Toluene : Ethyl acetate: Methanol [7:2:1]	0.60, 0.78, 0.88.
Methanol	Toluene : Ethyl acetate [7:3]	0.41, 0.61, 0.21, 0.50, 0.82.

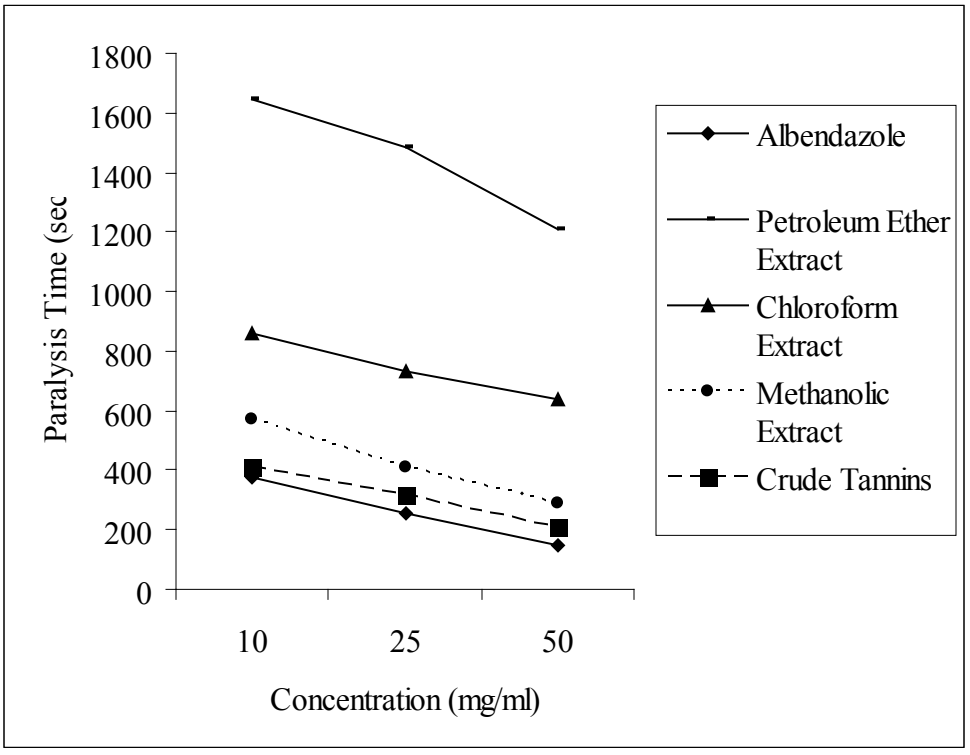

Figure 8. Death time for Anthelmintic activity of all extracts and crude tannins

indicating that crude tannins had some amount of phenol and flavonoids.

TLC analysis

Thin layer chromatography was performed for all the extracts and results were shown in Table 5. Crude tannins showed more than one spot on TLS plates. This may be due to presence of phenols and flavonoids along with tannins.

Evaluation of anthelmintic activity

Anthelmintic activity was evaluated for petroleum ether, chloroform, methanol extracts and isolated crude tannins. The results of paralysis and death time were shown in Table 6, Fig. 7 and 8. Among the extracts, methanolic extract showed very good anthelmintic activity. Methanolic extract had paralysed the earthworms within 9.3 min, 6.5 min and 4.5 min at the concentrations of 10 mg, 25 mg and 50 mg per ml respectively. Control was observed for more than 8 hrs and no paralysis and death were occurred. Crude tannins isolated from methanolic extract were evaluated for anthelmintic activity at various concentrations in mg per ml level. At all the concentrations, paralysis and death time of crude tannins were less than the all extracts. Paralysis and death times of crude tannins were very close to the paralysis and death times of Albendazole.

CONCLUSION

The present study on pharmacognostical evaluation on *Leucas indica* will provides useful information for its identification. Phytochemical analysis conform the presence of different phytochemicals in *Leucas indica*. The values of paralysis time and death time of methanolic extract as well as isolated crude tannins are very close to the values of albendazole. So the plant, *Leucas indica* possess anthelmintic activity. Future plan of work includes purification and characterization of isolated tannins from *Leucas indica*.

ACKNOWLEDGMENTS

The authors sincerely thank Dean and Principal of Faculty of Technology, Osmania University and also the management of Nalanda College of Pharmacy for their support in successful completion of the above work.

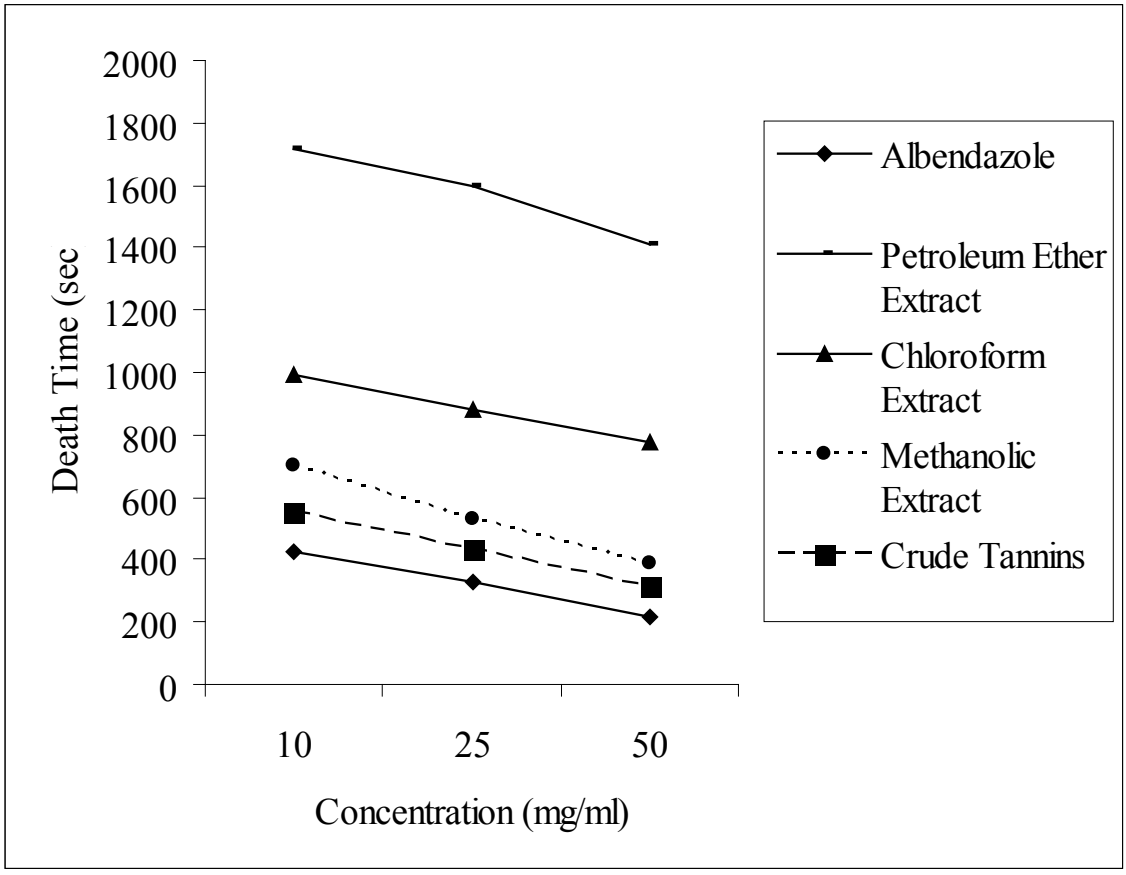

Figure 7. Paralysis time for Anthelmintic activity of all extracts and crude tannins

Table 6: Anthelmintic activity of entire plant powder of Leucas indica

Extract	Concentration (mg/ ml)	Time in minutes (Mean ± SEM where n=3)	
		For paralysis	For death
Control (5% Dimethyl formamide in Saline solution)		-----	-----
Standard Albendazole	10	6:16 ± 0.01	7:08 ± 0.034
	25	4:15 ± 0.011	5:30 ± 0.015
	50	2:28 ± 0.011	3:37 ± 0.0142
Petroleum ether	10	27:28 ± 0.020	28:36 ± 0.026
	25	24:42 ± 0.022	26:37 ± 0.013
	50	20:07 ± 0.06	23:34 ± 0.031
Chloroform	10	14:18 ± 0.07	16:35 ± 0.029
	25	12:15 ± 0.01	14:38 ± 0.017
	50	10:37 ± 0.046	12:55 ± 0.033
Methanol	10	9:31 ± 0.030	11:38 ± 0.027
	25	6:52 ± 0.048	8:53 ± 0.034
	50	4:50 ± 0.018	6:25 ± 0.02
Crude tannins	10	6:52 ± 0.017	9:11 ± 0.008
	25	5:13 ± 0.012	7:13 ± 0.020
	50	3:27 ± 0.020	5:10 ± 0.003

"----"indicates no paralysis and death occurred even after 8 hrs.

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Fatty acid composition of seed oil from some *Cleome* species

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ABSTRACT

Present paper describes seed oil fatty acid composition of *Cleome viscosa, C. simplicifolia, C. gynandra, C. chelidonii* and *C. speciosa*. In most of these species linoleic and hexadecanoic acids were found to be prominent. However, palmitic acid was predominant in *Cleome viscosa*.

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INTRODUCTION

Oil is any substance that is liquid at ambient temperatures and is hydrophobic but soluble in organic solvents. All oils can be traced back to organic sources. Most naturally occurring fatty acids are of the *cis* configuration, although the *trans* form does exist in some natural and partially hydrogenated fats and oils. Oils are liquid form esters of fatty acids[1]. Fatty acids are Lipids which are insoluble in water but soluble in organic solvents. Fatty acids are C, H, and O compounds, carbon chain skeletons with (C-COOH) carboxylic group at one end. Generally fatty acids are of two types, saturated and unsaturated fatty acids.

Common examples of unsaturated fatty acids present in seed oil are Myristoleic acid, Palmitoleic acid, Sapienic acid, Oleic acid, Linoleic acid, Erucic acid, Docosahexaenoic acid etc. Saturated fatty acids are Propanoic acid, Butanoic acid, Pentanoic acid, Hexanoic acid, Heptanoic acid, Octanoic acid, Nonanoic acid, dodecanoic acid, Tetradecanoic acid, Pentadecanoic acid, Hexadecanoic acid, Heptadecanoic acid, Octadecanoic acid, Nonadecanoic acid and Eicosanoic acid.

Gabriel *et al*.[2] studied fatty acid constituents present in the seed oil of *Cleome viscosa*. Uma Devi and Zaidi[3] studied fatty acid composition of *Cleome icosandra* and they observed that seeds contain 26% of oil. The oil contains only a small amount of saturated fatty acids and high amounts of unsaturated fatty acids.

An attempt was made to estimate oil and its fatty acids composition in seeds of some additional *Cleome* species.

MATERIALS AND METHODS

Seeds of *Cleome* species were homogenized and extracted in petroleum-ether and crude oil content as estimated according to the method by Sadasivam & Manikam[4]. The seed oil was further used by adding 1:1 methanol for GCMS analysis for fatty acid composition[5]. In GCMS study peaks of different fatty acids (according to retention time and percent area) were obtained which were compared to those for standard fatty acids from the library of GCMS.

RESULTS AND DISCUSSION

The GCMS analysis of fatty acids in the oil from seeds of *Cleome* species has been recorded in Table 1 and Fig. 1. It is found that all *Cleome* species contained Octadecadienoic and Hexadecanoic acids (commonly) at higher concentration (Table 1). In *Cleome viscosa* 49.66% hexadecanoic acid (ethyl and methyl esters together) was found with 48.85% ethyl ester of hexadecanoic acid. According Gabriel *et al*.[2] fatty acid esters especially ethyl palmitate is a major constituent present in the seed oil of *Cleome viscosa*. Ethyl palmitate is nothing but a form of hexadecanoic acid. In other species seed oil it was secondary major compound e.g. in *Cleome simplicifolia,* 17.33%; *Cleome chelidonii,* 21.08%; *Cleome gynandra,* 24.57% and in *Cleome speciosa* it was 24.59%.

In all these species hexadecanoic acid was found in various forms such as ethyl ester, methyl ester, Hydroxy methyl or 1, 2 ethanediyl ester. However, in the remaining four species hexadicanoic acid was found in measurable amount (Table 1). In seed oil of all *Cleome*

Figure: Gas chromatographs of fatty acids in the seed oil of different *Cleome* species

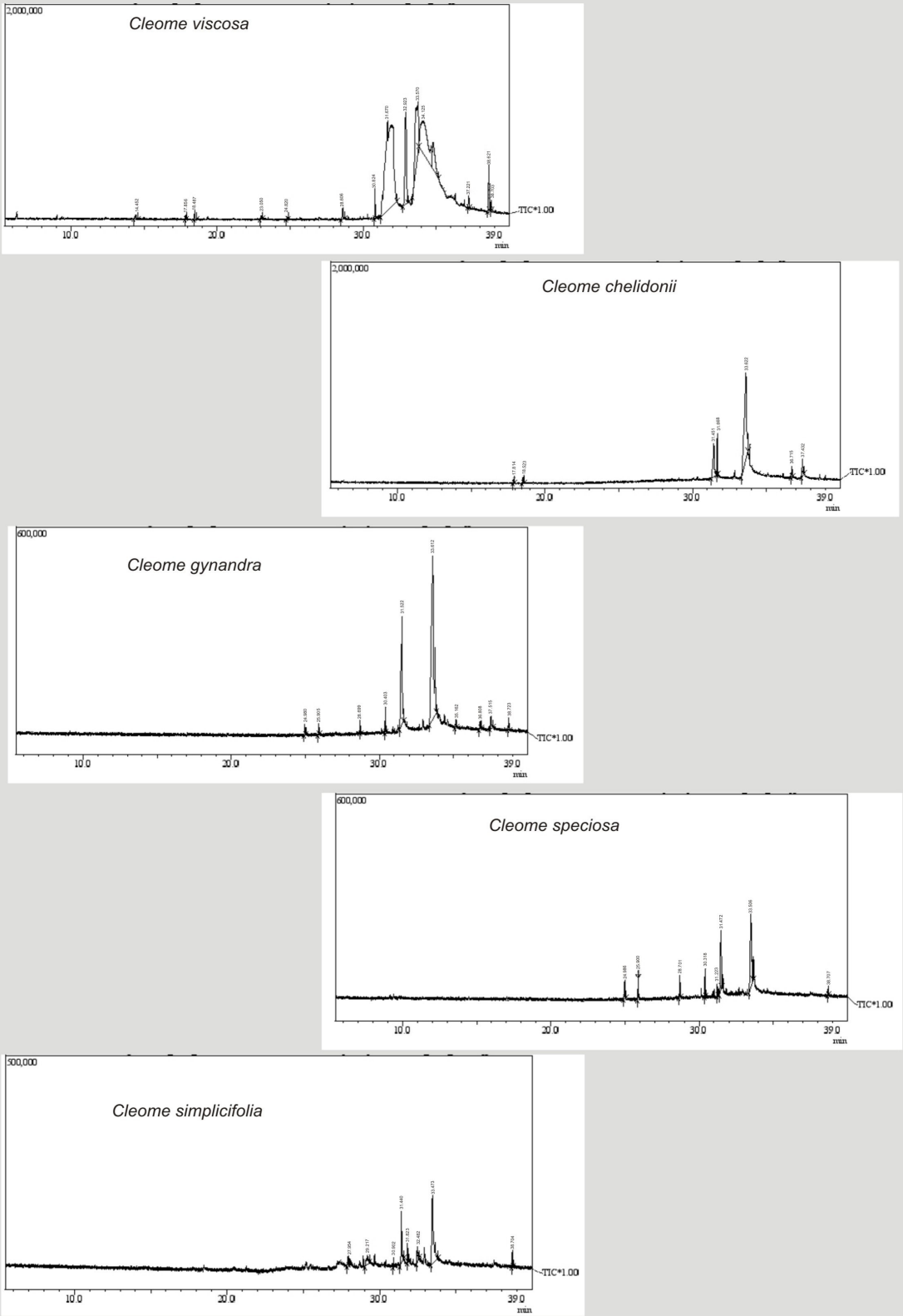

Figure 1. Gas chromatographs of fatty acids in the seed oil fo different *Cleome* species

Table 1: Fatty Acids composition of the Seed Oil from Different *Cleome* Species

Fatty acid		Concentration (%) in				
		Cleome viscosa	***Cleome simplicifolia***	***Cleome chelidonii***	***Cleome gynandra***	***Cleome speciosa***
Octanoic Acid		0.36	–	–	–	–
(Z)6,(Z)9-Pentadecadien-1-ol		–	–	4.56	–	–
1,2-Benzene-dicarboxylic acid,	bis(2methylpropyl) ester	–	–	–	1.85	5.83
	di isooctyl ester	0.35	4.99	–	1.36	2.30
2,4-Decadienal		0.47	–	1. 67	–	–
9,12-*Octadecadienoic acid* (Z,Z)		22.26	74.06	–	68.07	51.29
	1-methylethyl ester	0.52	–	–	–	–
	2-hydroxy-1-(hydroxymethyl)ethyl ester	–	–	2.11	–	–
	methyl ester	10.42	3.63	–	–	–
	ethyl ester	12.66	–	70.59	–	–
9-Oxononanoic acid		0.21	–	–	–	–
Cyclopentadecanone, 2-hydroxy		–	–	–	–	4.22
Diethyl Phthalate		–	–	–	0.96	–
Dodecanoic acid		0.08	–	–	–	–
Hexadecanoic acid	n	–	15.56	15.47	23.78	24.59
	ethyl ester	48.85	–	5.61	–	
	methyl ester	0.81	1.77	–	–	
	1(hydroxymethyl)-1,2-ethanediyl ester	–	–	–	0.79	–
Lauric anhydride		–	–	–	1.12	7.04
Pentadecanoic acid		–	–	–	–	–
Propyleneglycol monoleate		–	–	–	0.84	–
Tetradecanoic acid		0.52	–	–	1.23	6.23
Z-6,17-Octadecadien-1-ol acetate		2.48	–	–	–	–

species except *Cleome viscosa*, 9–12 Octadecadienoic acid was the major fatty acid found in various ester forms such as methyl-ethyl ester, 2 hydroxy-1-(hydroxymethyl)ethyl ester, methyl ester and ethyl ester as shown in Table 1. Concentration of this fatty acids in *Cleome* species varied from about 45 to 78%, the highest concentration being 77.69% in *Cleome simplicifolia* and that lowest (45.86%) in *C. viscosa*.

In seed oil of all these species 9,12-Octadecadienoic acid (Z,Z) was predominent while its ester forms were found in less amounts. However, in *Cleome chelidonii*, 9,12-Octadecadienoic acid ethyl ester was present at the highest level i.e.70.59% as shown in Table 1.

In seed oil of all studied *Cleome* species 1,2 Benzenedicarboxylic acid was also present but at lower amounts and in *Cleome chelidonii* seed oil it was not found. In *Cleome viscosa* it was 0.35% only, in *Cleome simplicifolia*, 4.99% and in *Cleome gynandra,* 3.2% only. In *Cleome speciosa* however, it was found to be relatively at higher concentration (8.13%).

1,2 Benzendicarboxylic acid, in its ester forms, e.g. diisooctyl ester was common in seed oil of all *Cleome* species (except *Cleome chelidonii*) and bis (2 methyl propyl ester) was found in *Cleome speciosa* and *Cleome gynandra* seed oil. In *Cleome speciosa* it was at higher level i.e. 5.83%.

Tetradecanoic acid appeared to be common in three *Cleome* species i.e. *C. viscosa* (0.52%), *C. gynandra* (1.23%) and *C. speciosa* (7.04%).

Lauric anhydride which is structural derivative of dodecanoic acid (Lauric acid) was present in *Cleome gynandra* (1.12%) and in *Cleome speciosa* (7.04%). While, 2,4 decadienal was observed in *Cleome viscosa* as well as *Cleome chelidonii.*

In seed oil of *Cleome viscosa* other fatty acids and their derivatives were also present such as Octanoic acid, Oxononanoic acid, Dodeccanoic acid and Z-6,17 Octadecadien1-0l acetate (2.48%). In seed oil of *Cleome chelidonii*, (Z)6 (Z)9 pentadecaden 1-0l (4.96%) derivative of Pentadecanoic acid was also present. In *Cleome*

gynandra, Propyleneglycol monoleate (0.84%) and Diethyl Phthalate (0.96%) have been recorded. While in *Cleome speciosa* 2-hydroxy Cyclopentadecanone, (4.22%) was at low level.

In *Cleome viscosa* dodecanoic acid was found with a very low conc. (0.08%) while in *Cleome simplicifolia* 1,2 benzendicarboxylic acid diisooctyl ester was present at 4.99%,concentration as lower fatty acid and in *Cleome gynandra* it was tetradecanoic acid (0.84%) which was at the lower concentration.

On the basis of GCMS analysis, seed oil from five different *Cleome* species showed much more similarity. Some compounds found common in all studied species at measurable concentration may be taxonomically important for family Cleomaceae or genus *Cleome*. While some compounds were specific and were found only in single species. This character is also helpful for the study of variation among the species.

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Development and Evaluation of Polyherbal Antidandruff Hair Oil

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ABSTRACT

The present study on formulated polyherbal Antidandruff hair oil is very effective in management of dandruff. However, during our experiment the formulation has proved to be excellent hair growth stimulators. Ideal base oil was selected by mixing of different proportions of two vegetative base oils. From the experiment it was reported that the volatile oils of *Eucalyptus globules* and *Ocimum gratissimum* along with the petroleum ether extract of *Hibiscus rosa sinensis*, *Phylanthus embelica, Tridax procumbens* posses antifungal activity. Further the last two extracts have significant hair growth activity. The result of open pilot clinical trials of prepared formulation brought strength to our claim. During the stability study of the formulation, the physical and chemical parameters remained unchanged after one month of preparation.

Keywords: Dandruff, Polyherbal hair oil, Antifungal, Open pilot clinical trials, Stability study.
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INTRODUCTION

Dandruff is the common complaints and is suffered by as many as 50% of the population of world at sometime during their life. The condition is generally characterized by the presence of flakes on scalp and in the hair. The symptoms can vary and the severity can range from mild scaling to severe scaling. Its prevalence and severity is greatest in young men and children. The older individuals suffer less frequently to dry scalp and dandruff .[1,2]

Among 800 male, 521 (65.1%) suffer with dandruff, whereas 279 (34.9%) don't suffer, particularly the person of age group 21-40 suffer most with dandruff than other age groups. [3] There are number of factors that responsible for the dandruff like fungal infections, hormonal imbalance, cold, dry weather, poor hygienic habits, long-term stress and anxiety, infrequent shampooing or inadequate rinsing of the scalp and hair, poor diet etc. [4] The central dandruff hypothesis remains that the lipophilic yeast *Malassezia furfur* (fig. 1) previously known as *Pityrosporum ovale* is the causal agent of dandruff. It is also known as an opportunistic pathogen involved in pityriasis versicolor, seborrheic dermatitis, *Pityrosporum* folliculitis, confluent and reticulated papillomatosis (Gougerot-Carteaud) and some kinds of atopic dermatitis.[1, 2, 5–7]

The common topical preparation used to treat dandruff includes- ketoconazloe, flucanazole, zinc-pyrthrone, selenium sulphide, coal tar etc. [5] Both synthetic and natural drugs are dispensed in different formulation like shampoos, cream, lotions, emulsions, hair oils and other cosmetic formulation.[8] Along with synthetic treatment, natural elements are more preferred for the management of dandruff.

MATERIALS AND METHODS

General

Poly herbal hair oil is an herbal product, formulated specifically for all ages of men, women & children. Herbal antidandruff hair oil is a perfect blend of ancient herbs that have been used from centuries to provide dandruff free scalp giving sufficient strength and vitality to hair. This product also provides all the necessary nourishment to the root of hair and promotes the natural growth of hair. It also checks and controls hair-fall, dandruff, thinning of hair and more.

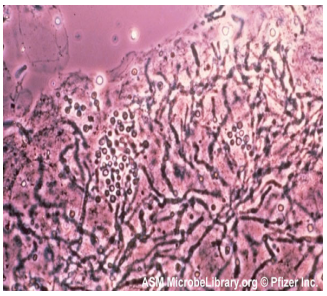

Figure 1. Lipophilic yeast *Malassezia furfur*

Taking mixture of ideal base oil, effective plant extract, some essential oils, color and perfumes help to formulate best antidandruff hair oil that can combat dandruff along with promotes hair growth[9].

Drugs of herbal origin have rapid demand for formulations. With rapid production of herbal formulations and coming up newer combinations of herbal drugs there is a need for standardization and evaluation for safer use. In this direction we have selected the formulated poly herbal hair oil for standardization both in analytically and pharmacologically.

Chemicals

0.5 alcoholic KOH, Wij's solution, saturated potassium iodide solution, solution of glacial acetic acid, chloroform, Folin-Denis regent, of 0.1N sodium thiosulphate solution ($Na_2S_2O_3$), 0.5 N HCl, 0.1 N NaOH solution, phenolphthalein indicator, fresh starch indicator.

Plant materials

There are some plant extracts, volatile oils and isolated compound found to be effective for treatment and management of dandruff (table 1). There are number of plants that proved scientifically to be effective against *Malassezia furfur*[10]. *Eucalyptus globules* Labil, *Wrightia tinctoria*, *Aloe vera, Cassia alata, Melaleuca alternifolia* (Tea tree oil), Azadirchta indica, *Phyllanthus emblica* L.. However, in Indian traditional medicines literature there are many plants which used traditionally for dandruff. [11–13]

Anti dandruff hair oil

The antidandruff hair oil enriched with natural oils prevents dandruff by eliminating microbial infections of the scalp. In the formulation, the plant species have been selected those used in Ayurvedic hair oils for the treatment of ailments of the head and scalp conditions. Systematic standardization (quality control) from raw materials to the final product the plants are proven to contain constituents that can cause hair growth and removing dandruff.

Base oil

Base oil or carrier oil play an important role in carrying and diluting highly concentrated essential oils. By means of dilution they inhibit evaporation rate of essential oils and spreading easily and evenly over the skin encourage quick absorption into the skin dermal layers. One part of coconut oil in the mixture with three parts of other suitable base oils will be ideal base oil. [14,15]

Components of the formulation

On the basis of literature survey, there can possible of formulating a poly herbal hair oil having antidandruff with hair growth stimulant activity.

Eucalyptus oil is obtained by steam distillation and rectification from the fresh leaves and terminal branches. It is colour less or pale yellow, it has aromatic and camphoraceous odour. [16, 17–22] The essential oil which is obtained from fresh leaves of *Ocimum gratissimum* by hydro distillation, the yield is about 0.69%. [23, 24] Hibiscus possesses antifungal property. [25] It is used in management of dandruff[26] Leaves and flowers are good for healing ulcers and for promoting growth and colour of hair. [27] Amla helps in good growth of hairs that's the region most of the marketed herbal hair oils contain amla as chief

Table 1: Parts of plants used for treatment and management of dandruff

Name	Botanical name	parts used
Eucalyptus oil	*Eucalyptus globules*	Fresh leaf
Ocimum oil	*Ocimum gratissimomum*	Fresh leaf
Hibiscus	*Hibiscus rosa sinensis*	Leaf
Amla	*Phyllanthus emblica*	Fruit
Tridax	*Tridax procumbens*	Entire aerial part
Mixture of base oils	*Cocos nucifera and sesamum indicum*	Vegetable oils

ingredients. The pharmacological properties of the amla are numerous. It has a wonderful antioxidant, antifungal, antibacterial etc. [28, 29] In Ayurvedic tridax is used as hair growth stimulators.[30,31] The juice of its leaves possesses antiseptic, insecticidal and parasiticidal properties. Coconut oil softens the hairs, imparts a luster, doesn't cause stickiness, light and penetrating oil.[2,32] Sesame oil obtained from the dried seeds of *Sesamum indicum*. Applying sesame oil brings darkness to hair. It may be used for hair and scalp massage. It is believed to reduce the heat of the body and thus helps in preventing hair loss.[33]

EXPERIMENTAL I

Screening for Antifungal activity

Required strain of *Malassezia furfur MTCC1374* tested in the growth medium like modified Emmons's Saboraudud's agar medium. Fluconazole was used as standard drug for the comparison of antifungal activity. Stock solutions of each 10 ml were prepared in sterile dimethyl sulfoxide (DMSO) at a concentration of 1mg/ml. Both solid and liquid Emmons's modifications of Saboraudud's medium were used for the study. Activation of culture, inoculums and media has preparared. [34]

Cup-plate/ Cylinder-plate method

The cup plate methods [35] for each Extract were done in duplicate methods and the average diameter (zone of inhibition) value has shown (fig. 2 and table 2).

Disc diffusion methods for Volatile oils

It was found that the antifungal assay of volatile oils *O. gratissimum* and *E. globulus* couldn't complete successfully in disc-plate methods as volatile oils didn't diffuse in the agar media. The antifungal screening of these volatile oils was done by disc diffusion methods.[9,36] The average diameter (zone of inhibition) value (fig. 3 and table 3) of volatile oil was described. Fig. 4 shows the chat of zone of inhibition in mm of different extracts and volatile oils. Minimum inhibitory concentration (MIC) value of the extracts and volatile oils are also calculated (table 4 and fig. 5).

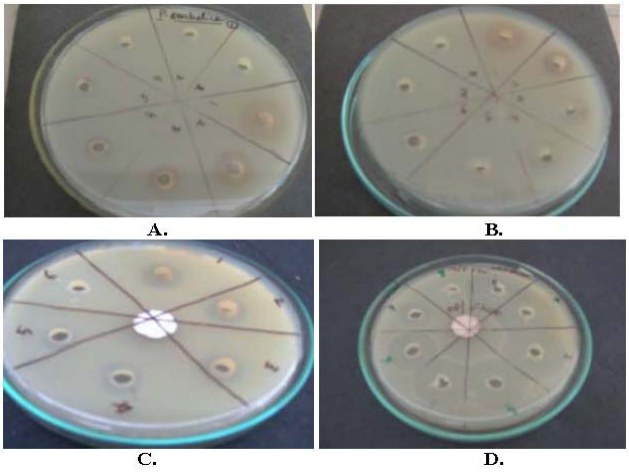

Figure 2. Zone of Inhibition (ZOI) of extracts: A: *Phylanthus embelica;* B: *Hibiscus rosa sinensis;* C: *Tridax procumbens;* D: *Fluconazole*.

Table 2: Zone of inhibition of extracts in compare to fluconazole

	Zone of inhibition (Diameter in mm) Concentration in mcg/ml.							
EX	**2000**	**1000**	**500**	**250**	**125**	**62.5**	**31.2**	**15.6**
PE	11	10	8	6	--	--	--	--
TP	9	8	6	--	--	--	--	--
HR	7	6	--	--	--	--	--	--
F	NA	35	28	20	18	14	12	7

EX: Extract, PE: *Phylanthus embelica*, TP: *Tridax procumbens*, HR: *Hibiscus rosa sinensis*, F: Fluconazole, NA: not applicable.

Hair growth activity of extraction on mice

Twenty four mice (25-30g body weight) were used in this study. Animals were procured from Institutional animal house (Regd. no. 621/02/ac/CPCSEA) of Birla Institute of Technology, Mesra. All animals were kept in polyacrylic cages and maintained under standard housing conditions (room temperature 24-27°C and humidity of 60-65% with 12:12; light: dark cycles). Food was provided in the form of dry pellets and water *ad libitum*. The animals were allowed to get acclimatized to the laboratory conditions for 7 days before the commencement of the experiment. All experiments involving animals complies with the ethical standards of animal handling and approved by Institutional Animal Ethics Committee.

Treatment protocols

Group 1: Control rats received no treatment.
Group 2: This group was treated with 2% extract of *Hibiscus rosa- sinensis* in base oils
Group 3: This group was treated with 2% extract of *Tridax procumbens* in base oils
Group 4: This group was treated with only placebo (Base oil without any drugs)

Base oils (Sesame oil and Coconut oil in ratio of 7:3) are taken for the study. 2gm. of each extract *Hibiscus rosa sinensis* and *Tridax procumbens* was dissolved in 100

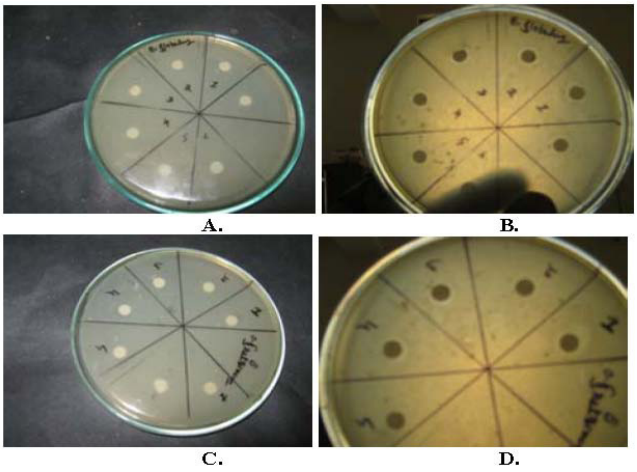

Figure 3. Zone of Inhibition (ZOI) of Volatile Oil: A and B: *Eucalypatus globules;* C and D: *Ocimum gratissimum.*

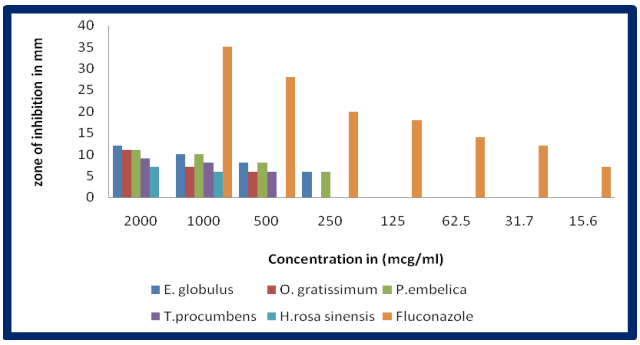

Figure 4. Chat of zone of inhibition in mm of different extracts and volatile oils

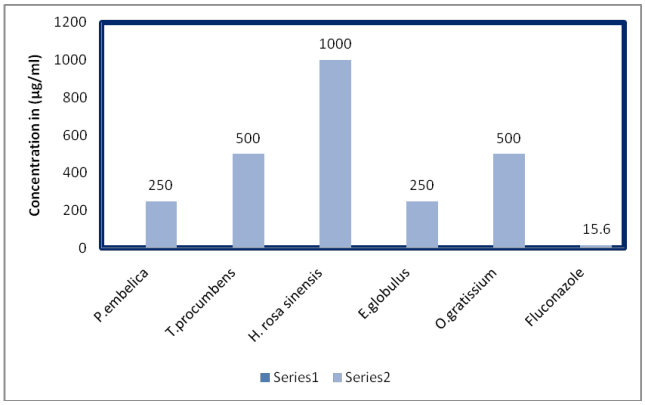

Figure 5. Chat of MIC value of the extracts and volatile oils

ml base oil to produce 2% active compound and it was further used for the evaluation of potential hair growth effects in vivo. 1 ml of the prepared oils of each extract was applied topically to the denuded area of each mice once a day.

Six animals in each group were taken, and shaved 2 cm^2 area of the hair from dorsal portions off all the rats was shaved off and wiped out with surgical sprits. One

Table 3: Zone of inhibition by volatile oil in compare to fluconazole

	Zone of inhibition (Diameter in mm) Concentration in mcg/ml.							
VO	**2000**	**1000**	**500**	**250**	**125**	**62.5**	**31.2**	**15.6**
EG	12	10	8	6	--	--	--	--
OG	11	7	6	--	--	--	--	--
F	NA	35	28	20	18	14	12	7

VO: Volatile oils, EG: *Eucalypatus globules*, OG: *Ocimum gratissimum*, F: Fluconazole, NA: not applicable.

Table 4: Minimum inhibitory concentration (MIC) value of the extracts and volatile oils

Extract and Volatile oils	MIC Values (µg/ml)
E.globulus	< 250
O. gratissimum	< 500
P.embelica	< 250
Hibiscus rosa sinenisi	< 1000
Tridax.Procumben	< 500
Fluconazole	< 15.6

ml of the prepared oils and the placebo were applied to the denuded area of the respective groups once a day and control group received no treatment, and this treatment was continued for 30 days.[27]

Hair was plucked randomly from the shaved area of shaved area from all mice, of each group on 15th and 30th day of treatment (fig. 6 and 7). The length of 25 hairs was measured and the average length of 25 hairs was determined. The result was expressed as the mean length ± S.D. of 25 hairs[37]

Results and Discussion

Hair growth was observed from the denuded area at the end of 1st week and the length of hair began to increase until the end of the treatment course. In comparison to the control, for all the groups the whole denuded area was covered with hair during the 4th week. Moreover, the hair growth was sparse in all the groups except the leaf extract-treated group and there was no considerable change in hair texture. It was found that the extract of *Hibiscus rosa sinensis* produce a significant growth with respect to the control and placebo. The groups those were treated with the extract of *Tridax procumbens* had also significant hair growth in comparison to the control and placebo group however lower than Hibiscus treated groups. The hair growth activity of the extracts was compared with the control and the placebo groups. Results were significantly

prepared with the corresponding control values and p values were calculated by Dunnett test.

Preparation of polyherbal formulation

From the above experiments it was found that the extracts and the volatile oils were effective against the *Malasezzia furfur* which is the main cause of dandruff. The 2% extracts of *Hibiscus rosa sinensis* and *Tridax procumbens* had showed good hair growth activity. So on the base of the MIC values of extracts, volatile oils and the extract concentration that showed hair growth activity, the hair oil was developed.

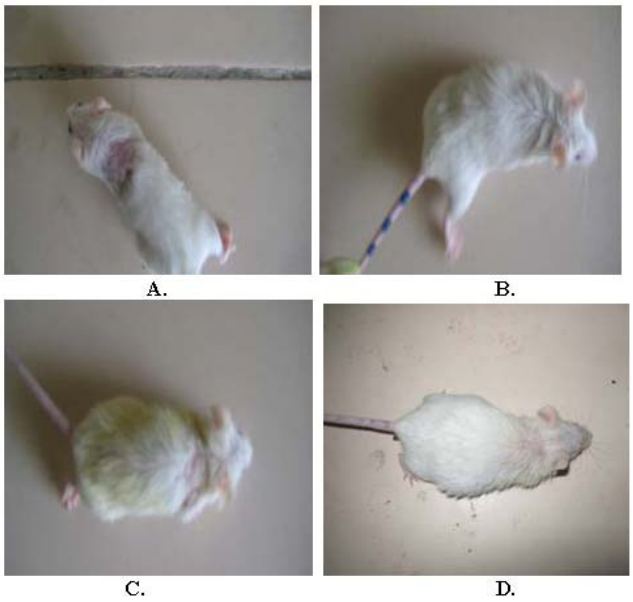

Figure 6. Growth of hair after 30th days; A: Control; B: Hibiscus extract treated; C: Tridax extract treated; D: Placebo.

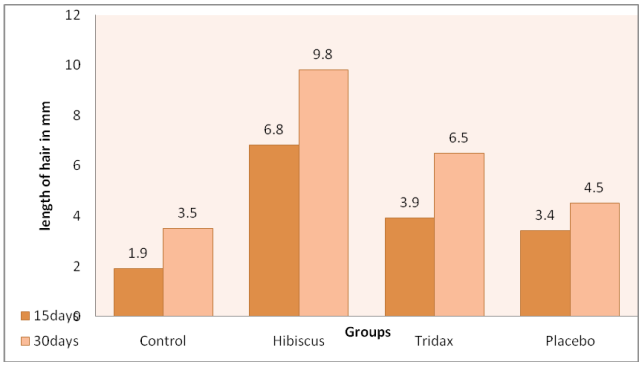

Figure 7. Chat of growth of hair in mice.

Table 5: Effect of different extracts on hair length of mice

Treated group	Length of Hair (mm)	
	15 days	30 days
Control	1.9 ± 0.09	3.5 ± 0.27
Hibiscus ext. treated	6.8 ± 0.33**	9.8 ± 0.25**
Tridax ext. treated	3.9 ± 0.11**	6.5 ± 0.05**
Placebo (Base oil)	3.4 ± 0.19**	4.5 ± 0.17*

Ext.: extract,
** p<0.01,
* p<0.05.

Formula

Each 100 ml contains

Ingredients	**Quantity**
Eucalyptus globulus (Volatile oil)	0.11 ml
Ocimum gratissimum (Volatile oil)	0.43 ml
Phylanthus embelica (Dried water extract)	0.1 gm
Tridax procumbens (Dried pet. ether extract)	2.0 gm
Hibiscus rosa sinensis (Dried pet. ether extract)	2.0 gm
Perfume	
Base oils (sesame oil + coconut oil)	Q.s

The base oil was taken in a glass vessel. Then it was allowed to boil, when the oil started to boil then extract of *phyllanthus embelica* was added to it and stirred continuously so that it was not allowed to adhere to the vessels. The dry petroleum ether extracts of hibiscus and tridax were added one by one with continuously stirring. Boiling of oil for long time was avoided, and then the oil was filtered through cloth twice. The require amount volatile oils like ocimum and eucalyptus were added after cooling. The perfume was added the end and stored in a glass container.[38]

Evaluation of formulated hair oil

The physical evaluation of formulated hair oil was observed in naked eyes and the colour of formulation was found to be dark green. The relative density of the formulated hair oil determined by using pycnometer was found to be 0.917 gm/ ml. Viscosity of a liquid measured by Ostwald viscometer was found to be 69.23 centipoise[39]. In the chemical evaluations, saponification values is 228, iodine value is 83.25, peroxide value is 5 and acid value is 1.2 were determined by the same procedure which followed for base oils. All the chemical parameters observation came within the range. The oil was kept in the bottle at room temperature for stability study.

EXPERIMENTAL II

Antifungal activity of formulation

After the successful formulation of hair oil, the antifungal (*Malassezia furfur MTCC1374*) activity was studied (fig. 8 and table 6).[9, 10, 36]

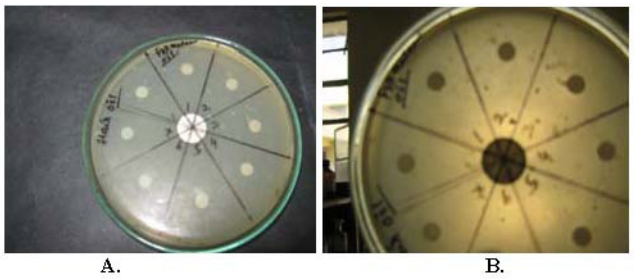

Figure 8. Zone of inhibition of hair oil in different concentration

Table 6: Zone of inhibitions of formulation

	Zone of Inhibitions (Diameter in mm)						
Screening	**Concentration in(µg/ml)**						
compound	**2000**	**1000**	**500**	**250**	**125**	**62.5**	**31.7**
Formulated oil	9	8	7	6	----	-----	-----

Hair growth activity of formulation on mice

The method for hair growth activity is studied in mice (table 7, fig. 9 and 10). The method is same as the previous method followed [27,37]

Allergic test of formulated hair oil

To know about the allergic and other adverse effect of the formulated hair oil open study was non-comparative, non-randomized which was conducted among some M. Pharma students of Department of pharmaceutical sciences, B.I.T, Mesra, Ranchi. Twenty five volunteers, from the age groups 22- 27 years, who were willing to give informed consent, were enrolled in the study. The formulated hair oil was applied on the lower surface of forehand and back side of ear pinna of twenty five volunteers (both male and female) .The volunteers were told not to wash the applied area for 24 hrs. After 24 hrs, no irritation, no allergic and no redness cases found among all the volunteers. It was concluded that, the formulated hair oil free from allergic and other skin related topical adverse effects.

Evaluation of antidandruff activity of polyherbal Hair oil [8,9]

An open pilot clinical study was planned to evaluate the clinical efficacy and safety. This pilot study was an open, non-comparative, non-randomized which was conducted among some M. pharma students and mess workers of B.I.T, Mesra, Ranchi. A total of 10 patients were enrolled in the study, there was a significant reduction in dandruff and itching at the end of 2nd weeks, similarly there was

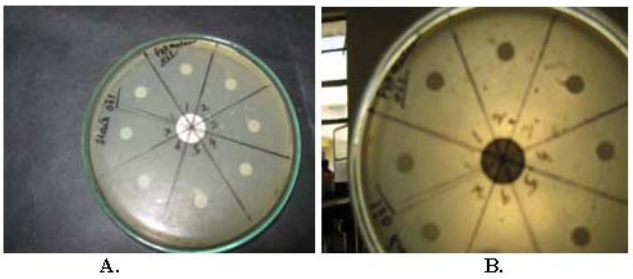

Figure 9. Hair growth activity in mice. A: Control group; B: Complete hair growth for animal treated with hair oil.

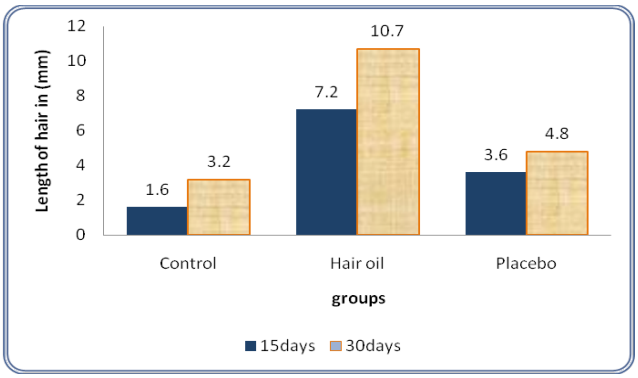

Figure 10. Chat of hair growth activity in mice treated with hair oil.

Table 7: Effect of formulation on hair length of mice

Treaded group	**Length of Hair (mm)**	
	15 days	**30 days**
Control	1.6±0.06	3.2±0.27
Formulated hair oil	7.2±0.39**	10.7±0.3**
Placebo (Base oil)	3.6±0.16**	4.8±0.11*

significantly reduction of white scales. In subjective evaluation, majority of the patients experienced remarkable overall improvement.

Stability study

After the chemical analysis the formulated hair oil was kept in a glass container at room temperature. The same oil again tested after the stipulated period. Physical evaluation of 30 days stored prepared oil, the colour found to be dark green, relative density is 0.916 and viscosity found to be 68.1 centipoise. In the chemical evaluation the formulation, saponification value is 239, acid value is 2.1, iodine value is 87.29 and peroxide value is 5.8. The stability study of the formulation, physical and chemical parameters remained unchanged after one month of preparation.

ACKNOWLEDGEMENTS

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Screening for Cytotoxic activity of Methanol Extract of *Putranjiva roxburghii* Wall (Euphorbiaceae) Seeds

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ABSTRACT

Screening of active compounds from plants lead to discover new medicinal drugs which have efficient protection and treatment roles against various diseases including cancer. The present study was designed to investigate the cytotoxicity of methanol extract of seeds of *Putranjiva roxburghii* Wall (Euphorbiaceae). The preliminary phytochemical screening of methanol extract showed the presence of phenols, alkaloids, saponins, steroids, flavonoids and glycosides. The extract showed cytotoxicity with LC_{50} of 427.74 µg/ml in brine shrimp lethality assay. Further studies on isolation of metabolites and their cytotoxicity are under investigation.

Keywords: *Artemia nauplii*, brine shrimp lethality, cytotoxicity, *Putranjiva roxburghii* Wall.

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INTRODUCTION

Bioactive compounds are almost always toxic in high doses. Pharmacologically is simply toxicology at a lower dose, and toxicology is simply pharmacology at higher dose. Thus *in vivo* lethality in a simple zoologic organism can be used as a convenient monitor for screening and fractionation in the discovery and monitoring bioactive natural compounds.[1] The brine shrimp lethality test is considered to be very useful in determining various biological activities such as cytotoxic, phototoxic, pesticidal, trypanocidal, enzyme inhibition, and ion regulation activities.[2–6] Recently, there has been interest in the brine shrimp lethality assay as a means of detecting ion regulation or ion-channel activity such as that involving Na^+-K^+-ATPase or calcium channels.[7–8] It can also be extrapolated for cell-line toxicity and antitumor activity.[2] The brine shrimp assay is very useful for the isolation of biogenic compounds from plant extracts.[9] This is a rapid method utilizing only 24 hours, inexpensive and needs no special equipment. It is so simple that no aseptic technique is required. It utilizes a large number of organisms for validation and a relatively small amount of sample. It does not require animal serum as needed for other methods of cytotoxicity testing.[10] This bioassay has been employed to determine cytotoxic activity of several plant extracts.

Putranjiva roxburghii Wall synonym *Drypetes roxburghii* (Wall.) Hurusawa belonging to the Family *Euphorbiaceae* is wild and cultivated throughout tropical India. It is known as Putrajivaka, Sutajva, Putrakamanjari in Ayurveda. The leaves, fruits and stones of fruits are given in colds and fevers, also in rheumatic affections. Rosaries, made of hard stones of the fruit, are placed around the necks of children to protect them from diseases. The seed kernel yield 0.5% of a sharp smelling essential oil of the mustard oil type. The oil contains isopropyl and 2-butyl isothiocyanates as the main constituents and 2-methyl-

butyl isothiocyanate as minor component. An additional glucoside, glucocleomin, has been found in the seed kernels. A glucosidic pattern similar to that in the seeds is reported in the shoots and roots. The fruit pulp contains a large proportion of mannitol and small quantities of a saponin glucoside and alkaloid. The alkaloid is also present in a small quantity in the stones of the fruit.[11] In a study, the oil of *Putranjiva roxburghii* exhibited the greatest toxicity against fungi and insect. The oil protected the peanut seeds for 6 months and did not exhibit any adverse effect on seed germination, seedling growth and general health and morphology of plants and thus was found to be potent preservative for peanut seeds against spoilage by fungi and insects during storage.[12] An ethnobotanical survey in Karandamalai, Southern Eastern Ghats of Tamil Nadu, a village inhabited by Valaiyans revealed the potent application of stem bark of *P. roxburghii* along with leaves of *Pterospermum suberifolium* on fractured bones.[13] In a study, changes in the contents of oil and fatty acids of *P. roxburghii* seeds obtained from different maturity stages was investigated and oleic and linoleic acids were found as abundant compounds.[14] Antinociceptive, antipyretic, and anti-inflammatory activities of *P. roxburghii* leaf extract in experimental animals were investigated. The ether extract dose-dependently produced analgesic activity in acetic acid-induced writhing in mice. At the dose of 400 mg/kg, the extract significantly decreased fever induced by yeast in rats. The extract exhibited inhibitory activity in carrageenin-induced paw edema in rats, croton oil-induced ear edema in mice and anus edema induced by croton oil at the high dose of 800 mg/kg in rats.[15] Analgesic and CNS depressant property of methanol extract of seeds of *P. roxburghii* Wall was investigated in mouse model. In analgesic activity, the reaction time increased significantly for the extract and standard groups when compared to the pre-drug treatment. The locomotor activity count in the extract and standard drug treated group was significantly reduced when compared to control group.[16] The antioxidant efficacy of methanol extract *P. roxburghii* was investigated by DPPH free radical assay, ferric reducing assay and hydroxyl scavenging assay. The extract showed potent activity in a dose dependent manner.[17] The present study was carried out to determine the cytotoxic activity of methanol extract of seeds of *P. roxburghii* by Brine shrimp lethality bioassay.

MATERIALS AND METHODS

Collection and Identification of Plant Material

The seeds of *P. roxburghii* (Voucher no. PK/SRNMN/Pr-301) were collected from the local supplier of Udupi city, Karnataka, authenticated in the Department of Botany and the voucher specimen was deposited in the department for future reference.

Extraction and Phytochemical Analysis

About 50 g of the powdered seed material was taken and added to 100 ml of methanol. The mixture was shaken for 30 minutes and left at room temperature overnight. The extract was filtered over Whatman No 1 filter paper, and the filtrate was concentrated under reduced pressure to pasty mass.[18] The methanol extract was subjected to chemical tests to screen the presence of various secondary metabolites.[19]

Cytotoxic Activity of Methanol Extract by Brine Shrimp Lethality Test

Methanol extract at concentrations 1, 10, 100 and 1000 µg/ml were used. The brine shrimp lethality test was conducted according to the method of McLaughlin.[20] Brine shrimp *Artemia nauplii* eggs (Nihon Animal Pharmaceutical Inc., Tokyo, Japan) were hatched in a container filled with air-bubbled artificial sea water which was prepared with 10 g of a commercial salt mixture (GEX Inc., Osaka, Japan) and 500 ml of distilled water. After 36-48 h, the phototropic shrimps were collected by pipette for bioassay. The different concentrations of methanol extract were tested in vials containing 5ml of brine and 25 shrimp in each of three replicates. Twenty five shrimps were transferred to each vial, artificial sea water was added to make up to 5 ml, incubated at 25°C and surviving shrimps were counted microscopically in the stem of the pipette against a lightened background after 24 hours. LC_{50} values greater than 1000 µg/ml for plant extracts were considered inactive (non-toxic).

RESULTS AND DISCUSSION

Preliminary Phytochemical Screening

The preliminary phytochemical analysis of methanol extract of *P. roxburghii* showed the presence of phenols, alkaloids, saponins, steroids, flavonoids and glycosides.

Brine Shrimp Lethality Assay

The result of cytotoxic activity of methanol extract in terms of mortality of Brine shrimps is presented in Table 1. The degree of lethality was found to be directly proportional to the concentration of the extract. Highest mortality took place in 1000 µg/ml concentration (72 % mortality) of extract where as no mortality was observed

"Table 1: Brine shrimp lethality data of methanol extract of *P. roxburghii*"

Conc. (µg/ml)	No. of shrimps	Mortality	% mortality	LC_{50} (µg/ml)[a]
1	25	0	0.00	427.74
10	25	0	0.00	
100	25	4	16.00	
1000	25	18	72.00	

[a]LC_{50} values determined by using linear regression using Origin 6.0 software.

at 1 and 10 µg/ml. LC_{50} of extract was 427.74 µg/ml and thus extract found to be toxic.

The brine shrimp assay is very useful tool for the isolation of bioactive compounds from plant extracts.[9] The method is attractive because it is very simple, inexpensive and low toxin amounts are sufficient to perform the test in the microwell scale. The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and anti-tumor properties.[20] The brine shrimp lethality assay is based on the ability of the extract to show lethality in laboratory cultured *Artemia nauplii* brine shrimp. It is considered as useful tool for preliminary assessment of toxicity. It has also been suggested for screening pharmacological activities of plant extracts.[21]

CONCLUSION

Although the brine shrimp lethality assay is rather inadequate regarding the elucidation of the mechanism of action, it is very useful to assess the bioactivity of the plant extracts. In the course of our studies, the brine shrimp lethality assay actually has proven to be a convenient system for monitoring biological activities. Even though, the present study on the crude extract is an addition to the scientific literature, detailed investigations on pharmacological activities and active ingredients could provide leads to interesting pharmaceuticals of plant origin.

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Wound Healing and Cytoprotective Actions of *Paullinia pinnata* L.

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ABSTRACT

Paullinia pinnata L. (Sapindaceae) is widely used ethnomedicinally in Ghana for the treatment of wounds and other skin diseases. In this study, the *in vivo* effect of the methanolic extract of *P. pinnata* on incision and excision wound models in rats were investigated, as well as the *in vitro* cytoprotective action of the extract on 142BR cell line against hydrogen peroxide-induced damage. The results of the *in vivo* wound healing tests showed that, upon administration of *P. pinnata* ointment, there was a decrease in the epithelialization period from 26.7 days (control) to 12.7 days along with a marked decrease in the scar area of 54.2 mm^2 (control) to 26.2 mm^2. There was also a significant increase in the tensile strength and hydroxyproline content compared to the control and comparable to nitrofurazone. The percentage closure of excision wound area showed that *P. pinnata* extract significantly stimulated wound contraction up to 69.2% in 7 days as compared to 46.2% given by the control and 67.5% by nitrofurazone. The *in vitro* cytoprotective studies revealed that different concentrations of the extract, applied simultaneously with 10^{-4}M hydrogen peroxide in the growth medium protected the human dermal fibroblast cells (142BR) against hydrogen peroxide-induced damage, the maximum being 86% at 50 µg/ml, almost comparable with that of catalase (control at 250 units/ml).

Keywords: *Paullinia pinnata*; wound healing; cytoprotective; oxidative damage.
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INTRODUCTION

Wounds are physical injuries that results in an opening or breaking of the skin. Proper healing of wound is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin.[1] Wound healing is a complex process characterized by inflammation, proliferation and migration of different cell types. In cutaneous tissue repair, oxidants and antioxidants play very important roles. Oxidants are known to have the ability to cause cell damage and may function as inhibitory factors to wound healing.[2] Natural products, particularly those of plant sources, provide a rich source of wound healing remedies in many cultures around the world where extracts, juices and exudates are used. Examples of these include *Ficus asperifolia*, *Gossypium arboreum* and *Chromoleana odorata*.[3] These plants, although diverse in terms of taxonomy, contain one or more active constituents that may influence wound healing.

Paullinia pinnata L. (Sapindaceae) is a tropical plant which is used traditionally in the treatment of infectious diseases, fractures and as an aphrodisiac.[4] It is also used in wound healing in the form of a poultice applied externally twice daily for a week in some parts

of Ghana.[5] Earlier work on *P. pinnata* has established its molluscicidal and antioxidant properties[6,7] while previous phytochemical investigations have shown the presence of triterpene saponins, catechol tannins, flavone glycosides, paullinoside A, paullinomide A, β-sitosterol and β-amyrin.[8–10]

As a follow up on our earlier report on the wound healing actions of *P. pinnata*,[11] we have investigated the effect of *P. pinnata* extract on excision and incision wound models *in vivo* and the cytoprotective effect of the extracts on human dermal fibroblast cell line [142BR].

MATERIALS AND METHODS

Plant materials

Plant materials were collected in the month of August, 2007 and authenticated at the Centre for Scientific Research into Plant Medicine (CSRPM), Akwapim-Mampong, Ghana, where voucher specimen (8/05/17) has been deposited.

Preparation of extract

200 gm of dried powdered root was packed into a cellulose thimble (28×100 mm) and soxhlet-extracted with 500 ml methanol over 48 hours until the material was exhausted, and the extract dried in vacuum to give a yield of 4.92 %w/w.

Animals used

Male Sprague-Dawley rats (160-180 g) were purchased from the animal house of the Central Drug Research Institute, Lucknow, India. They were kept at 26 ± 2°C and relative humidity of 44–55% and light and dark cycles of 10 and 14 hours respectively, for one week before the experiment. Animals were given the rodent diet (Amruth, India) and water *ad libitum*. All studies were conducted in accordance with the National Institute of Health's guidelines for Survival Rodent Surgery,[12] after approval from the institutional ethics committee. All surgical procedures were carried out under thiopentone sodium (25 mg/kg, i.p.) anaesthesia. Animals were allowed to recover and were housed individually in metallic cages containing sterilized paper cuttings.

In the experiment, the rats were divided into three groups (n=6). Group 1 was the control group which received simple ointment BP base, group 2 was treated with the reference standard (0.2% w/w nitrofurazone, a standard antimicrobial agent used in topical wound dressings), group 3 received *P. pinnata* ointment (33.3% w/w *P. pinnata* methanol extract in simple ointment BP) topically, on wounds created on the dorsal skin of rats daily, until the wounds completely healed.[13] 100 mg of ointment was spread over 500 mm^2 area.

Excision wound model

An impression was made on the dorsal thoracic region 1cm away from the vertebral column and 5 cm away from the ear using a biopsy punch (Acuderm, USA) of 2.5 cm diameter, on the anaesthetized rat. The skin of the impressed area was excised to its full thickness to obtain a wound area of about 500 mm^2. Haemostasis was achieved by blotting the wound with a cotton swab soaked in normal saline.

Wound area

Contractions, which contribute to wound closure in the first two weeks, were studied by tracing the edges of the raw wound. The wound area was measured after specific time intervals by retracing the wound on a millimeter scale graph paper. The difference in the area of the wound indicated the degree of wound healing.[14]

Collagen estimation

Hydroxyproline, which is a basic constituent of collagen was measured.[15] Tissues were dried in a hot air oven at 60-70°C to a constant weight and were hydrolysed in 6M HCl at 130°C for 4 hours in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4 M perchloric acid and colour was developed with the help of Ehrlich reagent at 60°C and absorbance measured at 557 nm using the Pye Unicam spectrophotometer.

Incision wound model

Rats were anaesthetized and two paravertebral long incisions made through the skin and cutaneous muscles at a distance of about 1.5 cm from the midline on each side of the depilated back of the rat. Full aseptic measures were not applied and no local or systemic antimicrobial was used throughout the experiment.[16] Each of the three groups of animals was treated in the same manner as for the excision wound model. No ligature was used for stitching. After the incision was made, the parted skin was kept together and stitched with black silk at 0.5 cm intervals. Surgical thread [No. 000] and a curved needle [No. 11] were used for the stitching. Continuous threads on both wound edges were tightened for good wound closure. The wound was left undressed and *P. pinnata*

extract [PP] ointment along with the water-soluble base ointment [control] and nitrofurazone ointment were applied topically twice daily for 9 days. When wounds were healed completely, the sutures were removed on the ninth day and tensile strength was measured with a tensiometer.

Tensile strength

The tensile strength of the healing tissues was measured using the method of Govindarajan.[17] The sutures were removed on the ninth day after wounding and the tensile strength measured on the tenth day. The animals were anaesthetized and healing tissue along with normal skin at two ends was excised for tensile strength measurement using Tensile Testing Machine TKG-20 [from Fine Testing Machines, India]. Strips of 8mm width and 20 mm length were cut out from the excised tissue in treated and control animals and were loaded between the upper and lower holder of the machine in such a way that the effective load bearing size was 8 x 8 mm with the wound remaining in the centre. The total breaking load was measured in Newtons and the tensile strength was calculated by the following equation;

Tensile strength = Total breaking load / Cross-sectional area

The *P. pinnata* ointment along with the standard and control were applied throughout the period, twice daily for 9 days. The mean tensile strength on the two paravertebral incisions on both sides of the animals was taken as the measures of the tensile strength of the wound for an individual animal. The tensile strength of *P. pinnata* ointment treated wounds was compared with the control and nitrofurazone ointment as the standard. Further epithelization period and scar area were measured daily for 25 days after tensile strength determination.[14]

Protection of fibroblast (142BR) cells against hydrogen peroxide-induced damage

The fibroblast cells were cultured in minimum essential medium (MEM) supplemented with 10% FBS, 1% penicillin/1% streptomycin and 1% L- glutamine and maintained at 37°C in a 5% CO_2-air atmosphere. Based on the growth profile, the optimal plating density of the cell line was determined to be 5000 cells/well, to ensure almost confluent growth during the period of the experiment. The optimal concentration of hydrogen peroxide that can induce a recoverable damage on fibroblast cells of concentration 5000 cells/well was determined using a modified assay procedure by Murrell.[18] Here, the culture medium was removed and cells exposed to different concentrations from 10^{-5}M to 10^{-3}M, of freshly prepared hydrogen peroxide in HBSS for 3 hours at 37°C. The cells were washed again with PBS and MEM containing 10% FBS added. The cell damage was evaluated using Neutral Red assay and also by visual examination. A concentration of $1x10^{-4}$ M was selected as the suitable dose of hydrogen peroxide because cells treated with this concentration of hydrogen peroxide were able to recover from damage and assume an almost perfect shape after treatment and incubation with MEM containing 10% FBS.

Cytoprotective action of plant extract on human skin fibroblasts

The method used for the hydrogen peroxide assay was the one described by Yamasaki *et al*.[19] and modified to evaluate the protective effect of the extracts on the cells against oxidant injury induced by hydrogen peroxide. Fibroblast cells were seeded at 5000 cells/well in a 96-well plate and incubated for five days until almost confluent. The growth medium was then discarded and the confluent cells subjected to three different types of experiments.

In the first experiment, the cells were pre-treated with different concentrations of the extract overnight, after which they were exposed to 10^{-4} M hydrogen peroxide in the standard growth medium and incubated for a further 3 hours.

In the second protocol, fibroblast cells were pre-incubated with the extract at different concentrations overnight, before exposure to the same concentrations of extract together with 10^{-4} M hydrogen peroxide in the growth medium.

In the third experiment, different concentrations of extract were applied simultaneously with 10^{-4} M hydrogen peroxide in the growth medium and incubated for 3 hours at 37°C. Catalase (250 unit/ml), an antioxidant enzyme was used as positive control in all experiments.

After the incubation period, the fibroblast cells were stained with Neutral Red and observed microscopically for cell damage, followed by the Neutral Red assay to quantify the degree of protection of fibroblast cells by the extract against hydrogen peroxide damage.

Statistical analysis

One-way ANOVA was used for the comparison of the means. Results are expressed as mean ± SD (standard deviation) data, using the GraphPad Prism Software, version 5.

RESULTS AND DISCUSSION

The results of the *in vivo* wound healing tests showed a decrease in the epithelization period from 26.7 days (control) to 12.7 days along with a marked decrease in the scar area of 54.2 mm^2 (control) to 26.2 mm^2 upon administration of *P. pinnata* ointment (Table 1). Significant increase in the tensile strength and hydroxyproline content was also observed as compared to the control and was comparable to the nitrofurazone.

The percentage close of excision wound area, which was an indication of degree of wound contraction, showed that *P. pinnata* extract significantly stimulated wound contraction with about 69.2% ($P<0.01$) in 7 days as compared to 46.2% given by the control and 67.5% by the nitrofurazone (Table 2).

The *in vitro* cytoprotective studies revealed the following results; the first and second protocols when fibroblast cells were pretreated with the extract to assess its effect on hydrogen peroxide induced damage, it was found by both microscopic examination and colorimetric assay that the cells were damaged (Fig. 1) and so were not protected. The cell damage however could be due to the inherent cytotoxicity of the extract rather than the effect of the hydrogen peroxide, due to the relatively high concentrations of the extracts used.

In the third protocol, different concentrations of extract were applied simultaneously with 10^{-4}M hydrogen peroxide in the growth medium and incubated for 3 hours at 37°C. Here, it was observed that fibroblast cells were significantly protected against hydrogen peroxide induced damage. The cytoprotective action was found to be 86% at 50 µg/ml, almost comparable with that of catalase (control at 250 units/ml) [Fig. 2].

1. Confluent fibroblast cells without any treatment. Cell density is thick and shapes look more elongated.
2. Fibroblast cells exposed to hydrogen peroxide only. There is reduced cell density and disruption of cell shape.
3. Fibroblast cells protected by catalase. Cell density is thick and cells are more elongated.
4. Fibroblast cells protected by extracts of *P. pinnata* against hydrogen peroxide damage. Cell density is high and cells look more elongated.

Flavonoids, tannins and triterpenoids have all been found in this specie and wound healing activity is often associated with these compound types. These active constituents promote the process of wound healing by increasing the viability of collagen fibrils and increase the strength of collagen fibres, either by increasing the circulation or by preventing the cell damage or by promoting the DNA synthesis.[20]

As the wound healing process involves various phases, the use of a single model is inadequate and no *in vitro* experiment exists that can collectively represent the various components of wound healing.[21] Because of this, *in vivo* assays are highly recommended to confirm the *in vitro* observations. Some of the *in vivo* assays of significance

Table 1. Effect of *P. pinnata* extract ointment on incision wound

Topical treatment	Epithelisation period (days)	Tensile strength (g)	Scar area (mm^2)	Hydroxyproline (mg/100mg tissue)
Control	26.7 ± 1.2	287.5 ± 17.3	54.2 ± 3.8	7.22 ± 0.34
PP ointment (100mg/500mm^2)	12.7 ± 1.1[b]	422.9 ± 19.8[a]	26.2 ± 3.4[a]	10.27 ± 0.45[c]
Nitrofurazone (2% ointment) [100mg/500mm^2]	11.5 ± 1.4[a]	428.2 ± 21.3[a]	27.9 ± 2.9[c]	11.7 ± 0.45[b]

Values are mean ± SEM for six rats

Statistically significant difference in comparison with control group:[a] $p<0.001$,[b] $p<0.01$,[c] $p<0.02$.

Table 2. Effect of *P. pinnata* extract ointment on excision wound

Topical treatment	Percentage of closed excision wound area after days			
	4	7	15	21
Control	27.4 ± 2.6	46.2 ± 3.1	66.3 ± 3.5	75.8 ± 3.6
PP ointment (100mg/500mm^2)	36.5 ± 2.9[a]	69.2 ± 3.7[a]	94.7 ± 4.8[b]	98.2 ± 4.1[b]
Nitrofurazone (2% ointment) [100mg/500mm^2]	37.2 ± 3.1[a]	67.5 ± 4.1[a]	96.9 ± 4.7[b]	99.3 ± 4.8[b]

Values are mean ± SEM for six rats.

Statistically significant difference in comparison with control group:[a] $p<0.01$,[b] $p<0.001$.

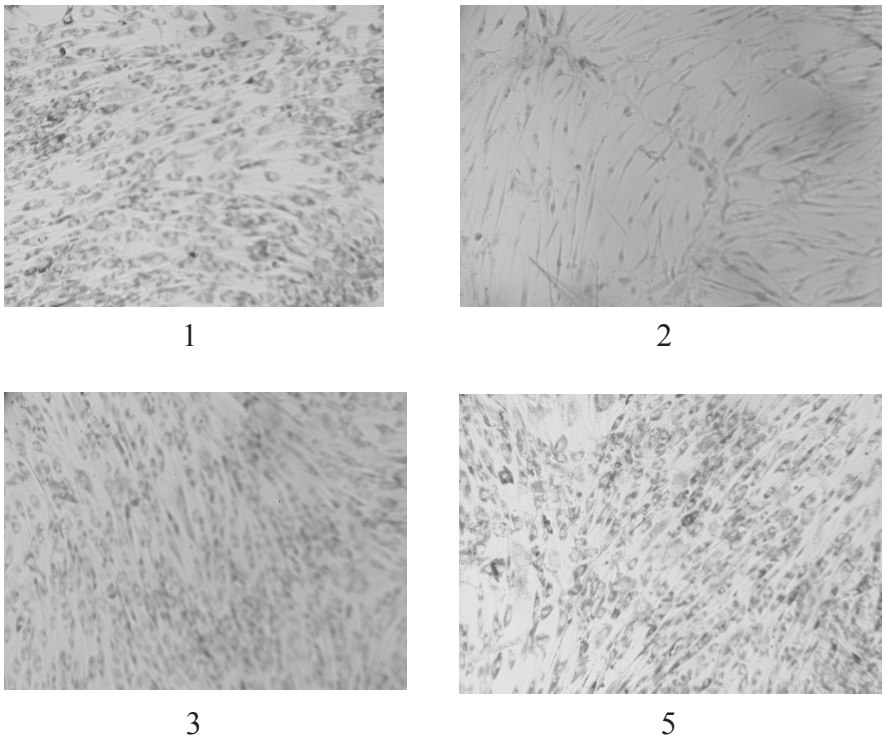
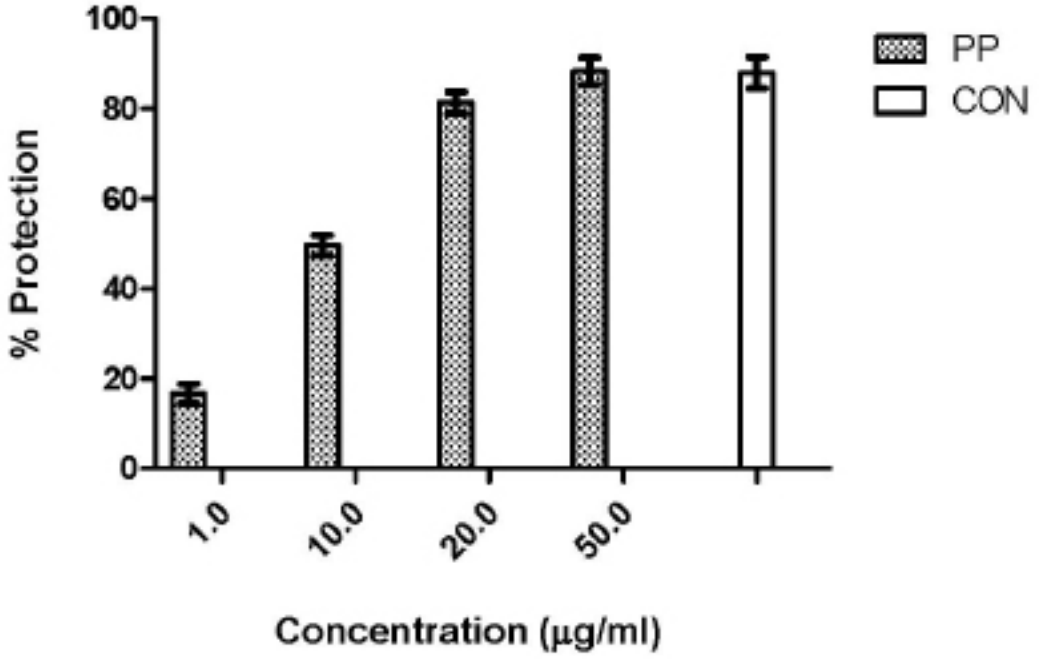


Figure 1. Photographic images of fibroblast cells after different treatments with hydrogen peroxide and extracts of *P. pinnata*



Key; PP- P. pinnata, CON- catalase (250 units/ml)
Figure 2. Protection of fibroblast cells against hydrogen peroxide-induced damage by simultaneous application of *P. pinnata* extract and hydrogen peroxide (10^{-4}M) [$p<0.01$]

include the determination of tensile strength, which is an indication of quality of the healing, and the determination of hydroxyproline content. Collagen is a major protein in the extracellular matrix and is the component that ultimately contributes to wound strength. Breakdown of collagen liberates free hydroxyproline. Measurement of

the hydroxyproline could therefore be used as an index for collagen turnover. In the present study, a significant increase in the hydroxyproline content of the granulation tissue of the animals treated with *P. pinnata* extracts was recorded compared to the control group, thus indicating the positive effect of the extract on collagen synthesis and hence, on wound healing. The increase in tensile strength of the granulation tissue indicated enhanced collagen maturation by increased cross-linking. The increase in tensile strength, as well as the epithelialization, could be attributed to the increased hydroxyproline content in the wound tissue.

The cytotoxicity of oxidants and the protective effects of the extract were indirectly assessed via cell viability. It was observed from the experiments with the fibroblast cells that pre-treatment of the cells with the extract gave no protective effect to the cells but rather caused damage to the cells due to the inherent cytotoxicity of the extract. However, the simultaneous application of the extract and oxidant (hydrogen peroxide) caused the cells to be protected to some degree. This is an indication that direct interaction of the extract with the oxidants (peroxide and superoxide) plays a major role in the protection of the cells *in vitro*. Furthermore, these observations suggest that the extract may not have caused any alteration to the cell membrane to protect it against oxidative damage.

CONCLUSIONS

P. pinnata extract has been shown to exhibit wound healing actions through both *in vitro* and *in vivo* models. The wound healing action is very significant and comparable to conventional wound healing remedy nitrofurazone, and the wound healing action may be attributed to the presence of phytochemicals such as flavonoids, tannins and triterpenoids that have been reported in this specie. These results lend support to the traditional usage of this species as a wound healing remedy.

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ABBREVIATIONS

CO_2	Carbon dioxide
FBS	Foetal bovine serum
HBSS	Hank' balanced salt solution
MEM	Minimum essential medium
PBS	Phosphate buffered saline

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A Study of Antimicrobial Activity of Few Medicinally Important Herbal Single Drugs Extracted in Ethanol, Methanol and Aqueous Solvents

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ABSTRACT:

There is a currently worldwide upsurge in the use of herbal preparations and active ingredients of medicinal plant in health care. This is particularly true in the rural areas of Asian countries where herbal medicines are the only choice for treating human ailments. Present study reveals the difference in the antimicrobial activity pattern of *Hemidesmus indicus, Smilax china,Ocimum basilicum, Ocimum sanctum, Ocimum cannum, Azadirachta indica A.Juss, Trigonella Foenum graceum* and *piper cubeba* extracted in Ethanol, Methanol and Aqueous solvents against the pathogenic organisms *E.coli* (ATCC-25922), *Staphylococcus aureus* (ATCC-25923), *Pseudomonas aeruginosa* (ATCC-27853),*Bacillus subtilis*(L10969), *Bacillus subtilis* (SL5740) and clinically isolated strains *Shigella, Klebsiella, Proteus, Salmonella paratyphi, Salmonella typhi and Stapylococcus aureus*. Among all the extracts Ethanol shows more activity with a zone of inhibition ranges from 10mm to 22mm. And Aqueous extract shows less inhibition which ranges from 6mm to 11mm.

Keywords: Anti-bacterial, Herbal drugs, Extracts
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INTRODUCTION

Infectious diseases caused by bacteria, fungi, parasites and viruses are still a major threat to public health, despite the huge progress in human medicine. Their impact is particularly large in developing countries due to unavailability of medicines and the emergence of widespread drug resistance [1]. Natural products especially, those used in ethno medicine provide a major source of innovative therapeutic agents for various conditions including infectious disease. The antimicrobial activity of different plant extracts have been reported by many authors [3,4,11]. Different classes of antibiotics have been used to control bacterial infections. However, the usefulness of existing antimicrobial agents is rapidly fading, tipping the balance in favour of multi-drug resistant pathogens, including MRSA and there appears to be few, if any, new classes of drugs currently in use to fight against the multi-drug resistant pathogens. Multidrug-resistance (MDR) exhibited by many bacterial species is a major problem in treating both hospital and community acquired infections.

Azadirachta indica A. Juss (Stem bark)has for long been used in the traditional Unani system of medicine for its beneficial properties. The aqueous extracts of stem bark used as tonic, stimulant and as a remedy against various skin ailments [6]. The chemical constituents are Nimbin, Nimbinin and Nimbidin.

Trigonella Foenum-Graecum (Fabaceae) found in nature and is cultivated in India and Pakistan is a well known medicinal plant having properties of reducing blood sugar level [10], antihelmentic, antibacterial [7], anti-inflammatory, antipyretic [5] and antimicrobial [2]. The important chemical constituents are saponins, coumarin, fenugreekine, nicotinic acid, phytic acid, Scopoletin and Trigonelline. *Hemidesmus indicus* (Asclepiadaceae) posses potent anti-inflammatory, anti-pyretic, anti-oxidant property[8], chemical constituents are Beta-Sitosterol, Beta-Amyrins, Lupeol, tannins and saponins.

MATERIALS AND METHODS

PLANTS MATERIALS

Bark of *Azadirachta indica A. juss* (Fabaceae) were collected from local area (Hyderabad, Andhra Pradesh ,India). Roots of *Hemidesmus indicus, Smilax china*, fruit of *Piper cubeba* and seeds of *Trigonella foenum graceum* were procured from local Herbal drugs dealers Hyderabad. Three species of *Ocimum- O.basilicum, O.sanctum, O.cannum* leaves were collected from Herbal garden CRIUM, Hyderabad. And were authenticated by Botanist at CRIUM Hyderabad ,A.P, India).

BACTERIAL CULTURES

Microorganisms tested in this study were *Escherichia coli* (ATCC 259220, *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (SL5740), *Bacillus subtilis* (L1O969), Clinical isolates - *Proteus, Salmonella paratyphi, Shigella, Salmonella typhi, Staphylococcus aureus and Klebsiella*.

EXTRACTION

The collected Herbal drugs were washed and air-dried for 48 h at the room temperature, chopped into small pieces and then soaked with three types of solvent at room temperature. The extract was filtered and then entire extract was concentrated to dryness using rotary evaporator under reduced pressure.

EVALUATION OF ANTI-BACTERIAL ACTIVITY

Anti-Bacterial activity of the extract was determined by Agar diffusion assay [9]. Bacterial strains were first grown in Mueller Hinton broth (MHB) under shaking condition for 24 h at 37°C and after the incubation period 0.1ml of the test the inoculums was spread evenly with a sterile glass spreader on Mueller Hinton Agar(MHA) plates. The seeded plates were allowed to dry in the incubator at 37°C, wells were made using sterile 6mm cork borer in the inoculated MHA plate. The wells were filled with 200µl of the extracts (re-suspended in respective solvents) .The concentration of stock extracts were 200 mg/ml. The inoculated plates were incubated at 37°C for 24 h. The plates were observed for the presence of inhibition of bacterial growth that was indicated by a clear zone around the wells. The size of the zones of inhibition was measured and the antibacterial activity was expressed in terms of average diameter of the zone of inhibition in millimeters. The results were compared with the standard antibiotics Ciprofloxacin (30mg). The photograph was taken in U.V-Visible documentation system.

RESULTS AND DISCCUSSION

The results of antibacterial activities in terms of zone of inhibition (mm) were presented in (Table 1). The antibacterial activities of single Herbal drugs extracts were tested against eleven bacterial strains. The results showed promising antibacterial activity against the bacteria tested. Among these Ethanol and methanol extracts were found to have a more potent inhibitory effect than Aqueous extracts. The observed activity may be due to the presence of potent phyto-chemical constituents in the drug extracts. Among all the drugs extracts of *Azadirachta indica* (Bark) and *Hemidesmus indicus* (Root) shows more activity on all the strains of bacteria. *Staphylococcus aureus* and *Salmonella paratyphi* was observed to be the most susceptible organism using Ethanol extracts of *Azadirachta indica* (Bark), at the same Ethanol extracts of *Hemidesmus indicus* shows no activity on *Salmonella typhi* and *Salmonella paratyphi*. Strains of *Bacillus subtilis* was found more susceptible to ethanol extracts of *Smilax china* (root). The Ethanol and Methanol extracts of *Tigonella foenum graceum* (seeds) were affective on *E.coli, Staphylococcus aureus* and *Salmonella typhi* and *Salmonella paratyphi* strains. Among the three *Ocimum* species extracts of Ethanol and Methanol of *Ocimum cannum* and *Ocimum sanctum* shows promising activity and least activity was found using Aqueous extracts. *E.coli, Staphylococcus aureus* and *Bacilus subtilis* strains showed good activity using *Piper cubeba* Ethanol and Methanol extracts compare with other bacterial strains. The susceptibility of tested Bacterial strains to Aqueous, Ethanol and methanol crude extracts of these Herbal drugs used is an indication of the potential of the extract as a drug that can be used against this organisms. *Ocimum basilicum* extracts and *Klebsiella* did not show any result in the entire test.

The phytochemical compounds are known to play an important roles in bioactivity of medicinal plants. Flavonoids exhibit as anti-inflammatory, anti-allergic,

Table -1: Antimicrobial activity pattern of few Herbal single drugs presented as diameter of Zone of inhibition.

ZONE OF INHIBITION (mm).																
		Hemidesmus indicus.			Piper cubeba			Smilax china			Azadirachta indica A. juss			Trigonella foenum graceum		
S.No	Micro organisms	Aq	Eth	Meth	Aq	Eth	Meth	Aq	Eth	Meth	Aq	Eth	Meth	Aq	Eth	Meth
1	E.coli (ATCC-25922)	0	14	16	N	13	12	0	11	0	13	16	14	0	14	17
2	Staphylococcus aureus (ATCC-25923)	0	12	12	N	13	14	0	14	12	14	20	16	0	18	16
3	Pseudononus aeruginosa (ATCC-27853)	14	0	0	N	11	0	0	13	12	10	21	9	0	15	14
4	Bacillus subtlilis (SLS740)	0	17	16	N	16	0	0	20	14	11	13	12	0	11	0
5	Baciillus substilis (L10969)	11	0	16	N	13	0	0	22	16	12	14	11	0	18	16
6	Proteus sp	0	15	14	N	12	0	0	0	10	12	13	11	0	0	0
7	Salmonella paratyphi	0	0	0	N	11	0	0	0	0	10	20	12	0	16	18
8	Shigella	0	14	13	N	10	0	0	0	13	15	19	18	0	8	11
9	Salmonella typhi	0	0	0	N	10	0	0	0	0	14	18	17	0	14	12
10	Staphylococcus aureus	11	14	13	N	13	11	0	14	12	15	17	18	7	7	11
11	Klebsiella	0	0	0	0	0	0	0	0	0	12	17	16	0	0	0

		Ocimum basilicum			Ocimum sanctum			Ocimum cannum			Standard antibiotic.
S.No	Micro organisms	Aq	Eth	Meth	Aq	Eth	Meth	Aq	Eth	Meth	Ciprofloxacin (30 mcg)
1	E.coli (ATCC-25922)	0	0	0	0	8	9	0	9	0	35
2	Slaphylococcus aureus (ATCC-25923)	0	0	0	6	11	11	0	11	10.5	32
3	Pseudononus aeruginosa (ATCC-27853)	0	0	0	0	0	12	0	10	12	35
4	Bacillus subtlilis (SLS740)	8	0	0	0	12	11	0	11	12	30
5	Bacillus subtilis (L10969)	0	0	0	6	9	16	0	11	9	31
6	Proteus sp	9	0	0	0	10	9	0	9	10	25
7	Salnonella paratyphi	0	0	0	6	0	11	9	0	0	31
8	Shigella	0	0	0	10	9	0	0	8	0	35
9	Salmonella typhi	0	0	0	7	0	10	6	0	0	30
10	Staphylococcus aureus	0	0	0	0	10	11	0	12	11	15
11	Klebsiella	11	0	0	0	0	8	6	0	0	20

analgesic and antioxidant as well. Rather than that flavonoids also exhibit a wide range of biological activities such as scavenge for hydroxyl radicals. The presence of the saponin compound in these herbal drugs which is supported the usefulness of these drugs in the managing inflammation. . The crude Herbal extracts tested were compared with control Ciprofloxacin (30mcg) Standard antibiotic. The Ethanol and methanol crude extracts showed the most significant zone of inhibition against almost all the bacterial strains.

CONCLUSION

As part of on going study to screen local plants for anti-bacterial activity *Hemidesmus indicus, Smilax china,*

Ocimum basilicum, Ocimum sanctum, Ocimum cannum, Azadirachta indica A.Juss, Trigonella Foenum graceum and *piper cubeba* were assessed for its actions against different strains of microorganisms extracted in Ethanol, Methanol and Aqueous solvents. We here report the effect of these three extracts on standard ATCC strains and few clinically isolated strains and also compared with standard antibiotic Ciprofloxacin respectively.

ACKNOWLEDGEMENT

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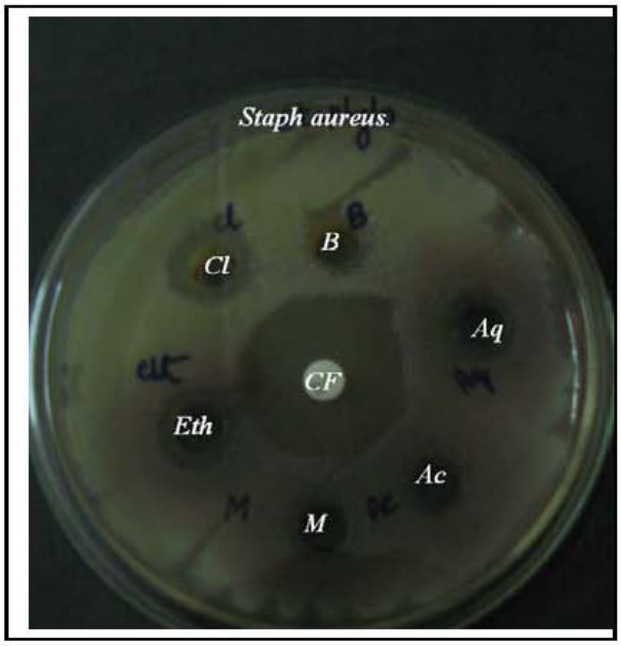
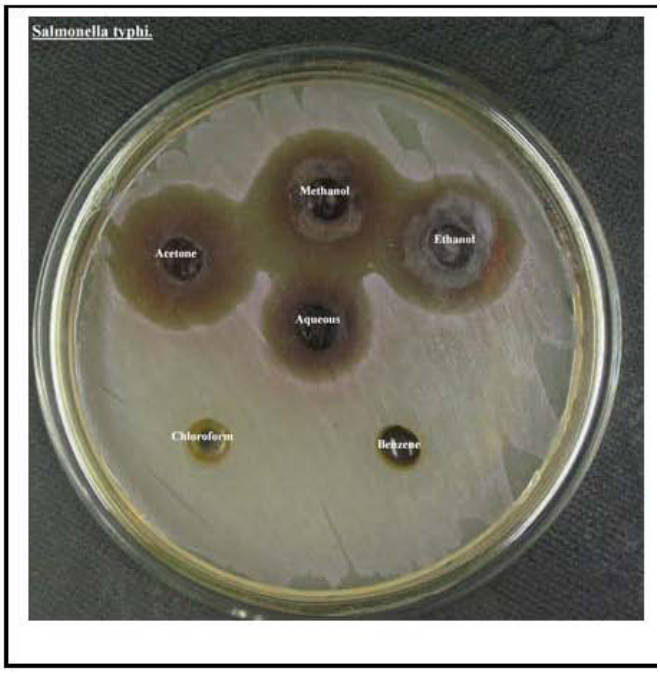


Azadirachta indica A.Juss



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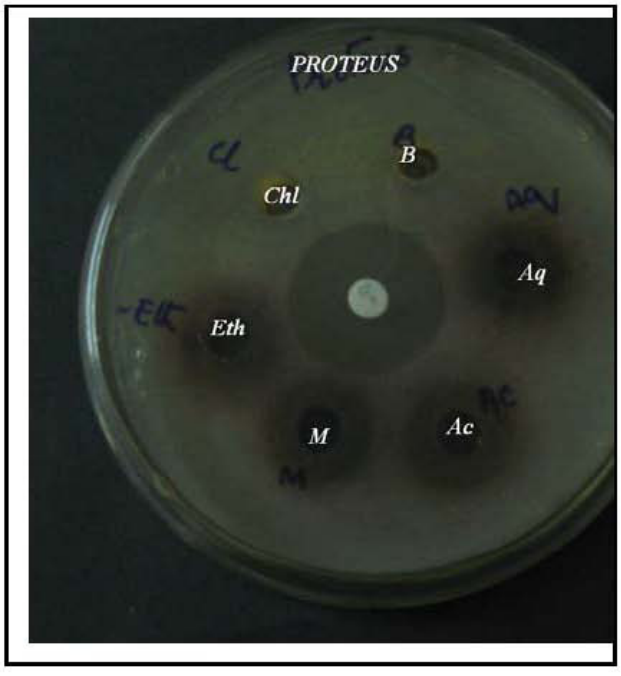

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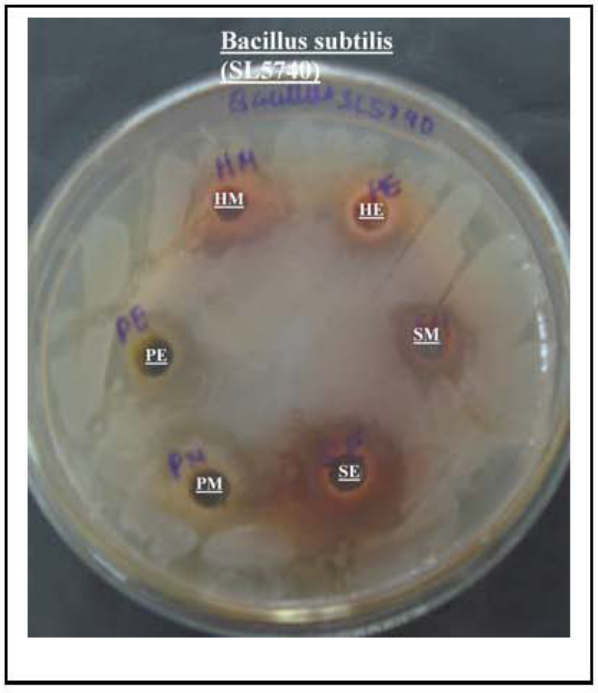
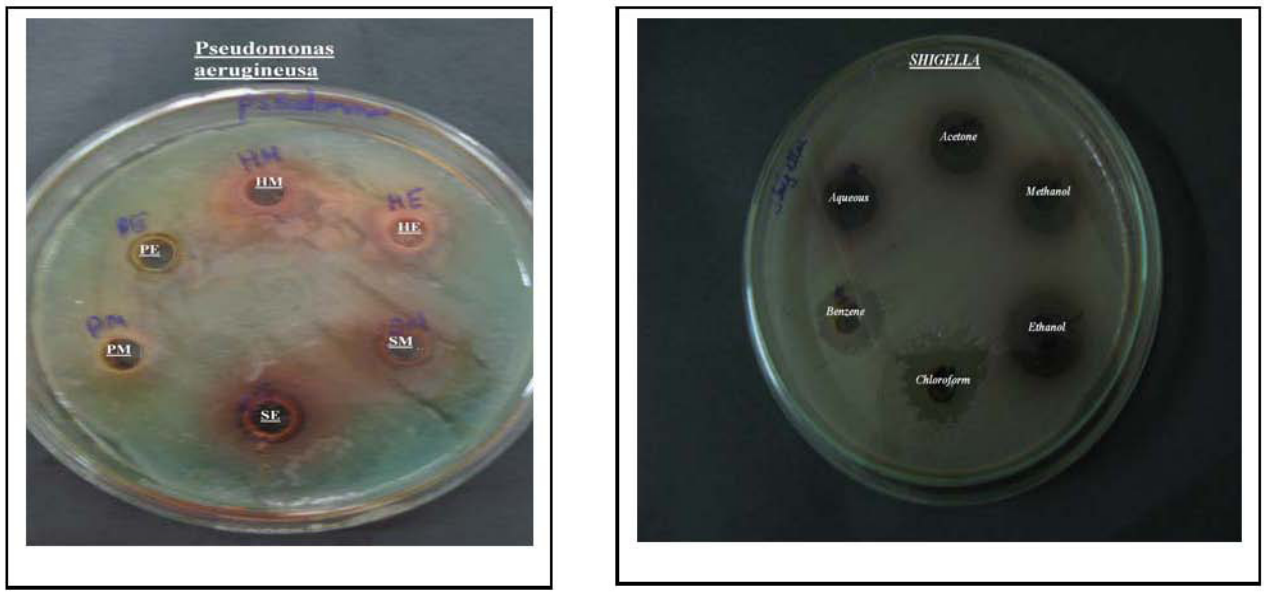
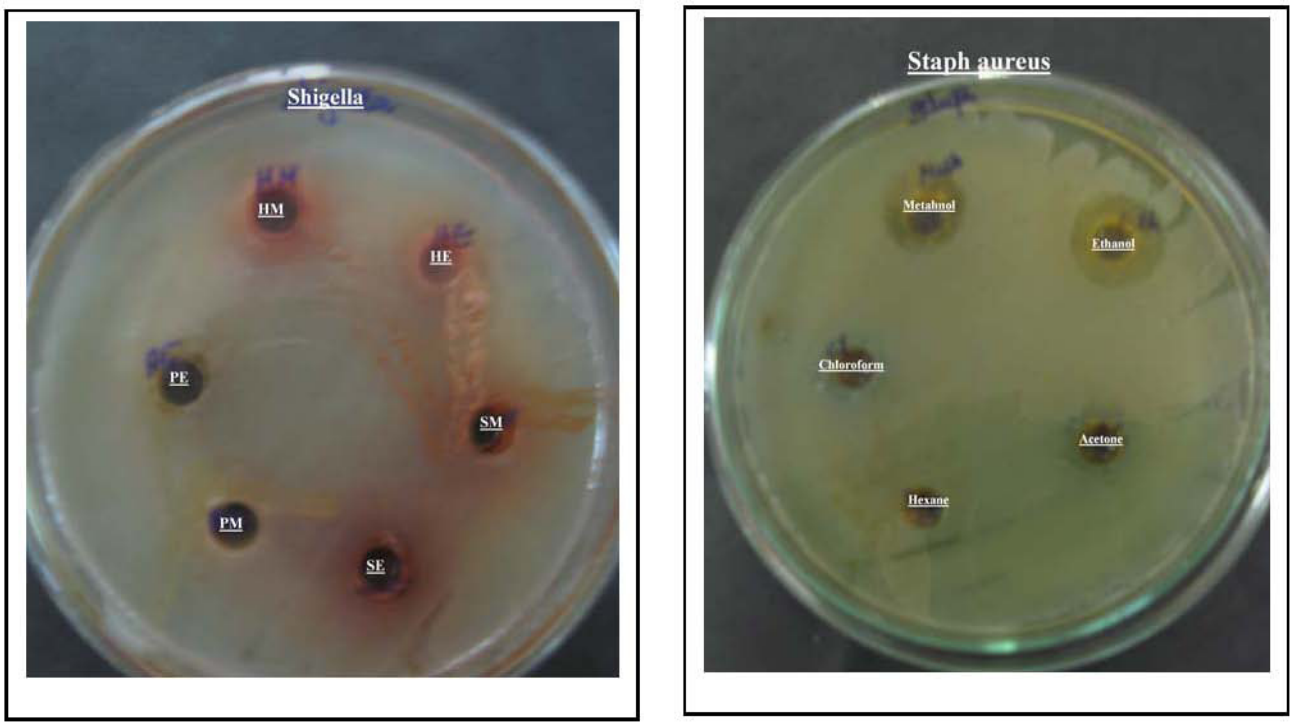




Azadiracht indica A.Juss



Trigonella foenum graceum

Figure 1: Photographs of Antimicrobial Activity of Herbal Drugs Used In The Study.

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In Vitro Antioxidant Studies of *Lagerstroemia speciosa* Leaves

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ABSTRACT

The hydro alcoholic extract of leaves of *Lagerstroemia speciosa* (LS) was studied for antioxidant activity on different *in vitro* models namely 1,1-diphenyl, 2-picryl hydrazyl (DPPH) assay, Hydrogen peroxide and Nitric oxide radical scavenging method, and superoxide readical scavenging by alkaline DMSO method. Various standards were also evaluated for comparison. The extract showed dose dependent free radical scavenging property in the tested models. *Lagerstroemia* showed IC_{50} value 4.75±0.25 µg/ml for DPPH method, which was comparable to that of ascorbic acid (IC_{50}=2.75±0.29 µg/ml) and rutin (7.89±0.51µg/ml) . For Hydrogen peroxide method, IC_{50} value was found to be 28.00±0.16 µg/ml, which compares favourable with Ascorbic acid (IC_{50}=187.33±3.45µg/ml) and rutin (35.26±0.166µg/ml). In Nitric oxide model, IC_{50} value was found to be 750±3.66µg/ml, which is very low when compared to Rutin (IC_{50}=68.5±2.27µg/ml). This study demonstrates the antioxidant activity of the *Lagerstroemia speciosa*.

Keywords: Antioxidant, *Lagerstroemia speciosa* (L.), free radicals, DPPH.
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INTRODUCTION

Free radicals oxidatively damage lipids and proteins and compromise genomic DNA integrity. They are widely recognised as the root cause of numerous degenerative diseases, including cardiovascular disease, cancer and aging.[1] Antioxidant compounds scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases.[2]

Lagerstroemia speciosa (Linn) Pers. (Lythraceae), also known as "Queens Crape Myrtle," is a deciduous tree which grow in tropical and subtropical areas. Native of Philippines it is commonly called 'banaba'. The banaba tree grows from 5 to 20 meters in height, and the leaves are smooth, acuminate, oblong, and 12 to 25 centimetres long.[3]

The leaves of Banaba (*Lagerstroemia speciosa* L.) has been used as a traditional oriental medicine to treat diabetes, polyuria and polydipsia.[4] It contains polyphenol compounds and corosolic acid.[5] Tea prepared from banaba leaves possesses antihypertension, diuretic, antiulcer activity.[6] The plant *Lagerstroemia speciosa* L. contains the monomeric and dimeric ellagitannins such as Lagerstoemin, reginin A[7], flosin B, reginine C and D.[8] Most of the reported biological activities and active constituents of this plant may be related to its antioxidant nature. Based on this idea the *in vitro* antioxidant activity of the extracts of leaves of *Lagerstroemia speciosa* has been evaluated and reported hereunder.

MATERIALS AND METHODS

Plant material

The leaves of *Lagerstroemia speciosa* were collected from the local market of Ootacamund, in the month of June 2006 and authenticated by Dr. Suresh Baburaj, Director, Survey of medicinal plants and collection unit, Ootacamund, Tamilnadu, India.

Chemicals

1,1-Diphenyl-2-picryl hydrazyl (DPPH) was obtained from Sigma Aldrich Co., St. Louis, USA. Naphthyl ethylene diamine dihydrochloride (NEDD) was from Roch – Light Ltd., Suffolk, UK. Ascorbic acid was obtained from Merck Ltd., Mumbai. Sodium nitroprusside solution (SNP), Nitro blue tetrazolium (NBT), Dimethyl sulfoxide (DMSO), Sulphanilic acid, Hydrogen peroxide, Glacial acetic acid were from Ranbaxy Laboratories Ltd., Mohali and SD Fine Chem., Mumbai, India. All chemicals and solvents used were of analytical grade.

Plant extract

The collected leaves were shade dried, coarsely powdered and extracted with 50% ethanol by cold maceration process. The extract was filtered and concentrated in vacuum and kept in a vacuum desiccator for complete removal of solvent. Hydro alcoholic extract of leaves of *Lagerstroemia speciosa* was obtained in the yield of 6.8%.

Characterization of plant extract

The identification tests for various phytoconstituents (alkaloids, glycosides, steroids, triterpenoids, tannins etc) of leaves of Lagerstroemia speciosa's hydro alcoholic extract were performed. The hydro alcoholic extract showed presence of alkaloids, glycosides, steroids, tannins, saponins, gums, mucilage, triterpenoids. (Table No. 1)

Preparation of Test and Standard Solutions

Hydro alcoholic extract of leaves of *Lagerstroemia speciosa* and the standard antioxidants (ascorbic acid and rutin) were dissolved in distilled dimethyl sulphoxide (DMSO) separately and used for *in vitro* antioxidant assays using four different methods. As DMSO interfere in the case of hydrogen peroxide method the extracts and the standards were dissolved in distilled methanol and used. The stock solutions were serially diluted with the respective solvents to obtained lower dilutions.

Table 1: Phytochemical Studies on hydro alcoholic extract of *Lagerstroemia speciosa* leaves

Tests	Inference *Lagerstroemia speciosa* Extract
Alkaloid	+
Tannins	+
Carbohydrate	+
Proteins & Amino acids	–
Steroids & Sterol	+
Glycosides	+
Saponins	+
Flavanoes & Flavonoids	+
Fixed oils & Fats	–
Triterpenoids	+
Gums & Mucilages	+

(+) Present , (–) Absent

Total Phenolic Compound Estimation

Antioxidant compounds generally contains phenolic group(s) and hence, the amount of phenolic compounds in the extract of leaves was estimated by using Folin-Ciocalteus reagent[9]. In a series of test tubes, 0.4 ml of the extract in methanol was taken, mixed with 2 ml of Folin-Ciocalteu reagent and 1.6 ml of sodium carbonate. After shaking, it was kept for 2 h reaction time. The absorbance was measured at 750 nm using a Shimadzu UV-160 Spectrophotometer. Using gallic acid monohydrate, standard curve was prepared and linearity was obtained in the range of 2.5 to 25 µg/ml. Using the standard curve the total phenolic compounds content was calculated and expressed as gallic acid equivalent in mg/g or % w/w of the extracts.

Free radical scavenging activity

The extract was tested for its in vitro antioxidant activity using standard methods. In all these methods, a particular concentration of the extract or standard solution was used which gave final concentration of 1000 µg/ml to 0.45 µg/ml after all the reagents were added. Absorbance was measured against a blank solution containing the extract or standards. A control test was performed without the extracts or standard. Percentage scavenging and IC_{50} values ± S.E.M (IC_{50} value is the concentration of the sample required to inhibit 50% of radical) were calculated.

DPPH radical scavenging activity

The assay was carried out in a 96 well micro titre plate. To 200 µl of DPPH solution, 10 ml of each of the test sample or the standard solution was added separately in wells of the micro titre plate. The final concentration of the test and standard solutions used are 1000 to 1.95 µg/ml. The plates were incubated at 37°C for 20 minutes and the absorbance of each solution was measured at 490 nm, using ELISA reader (Bio Rad Laboratories Inc, California, USA, Model 550).[10]

Scavenging of hydrogen peroxide radicals

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffer saline (PBS at pH 7.4). Various

concentrations of the extracts and standards in methanol (1ml) were added to 2 ml of hydrogen peroxide solutions in PBS. After 10 min, the absorbance was measured at 230 nm against a blank solution that contained extracts in PBS without hydrogen peroxide.[11]

Nitric oxide radical inhibition activity

The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (PBS, 1 ml) and 1 ml of extract in DMSO were incubated at 25°C for 150 minutes. After incubation, 0.5 ml of the reaction mixture containing nitrate was removed and 1 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was added, mixed well and allowed to stand for 5 minutes for completion of diazotization reaction, then 1 ml of naphthyl ethylene diamine dihydrochloride (NEDD) was added, mixed and allowed to stand for 30 minutes in diffused light at room temperature. The absorbance of these solutions was measured at 540 nm using ELISA reader against corresponding blank solution in a 96-well micro titre plate (Tarsons Product(P) Ltd., Kolkata, India) using ELISA reader (Bio Rad Laboratories Inc, California, USA, Model 550).[12]

Scavenging of Super oxide radical by alkaline DMSO method

To the reaction mixture containing 0.1 ml of NBT (1mg/ml solution in DMSO) and 0.3 ml of the extracts and standard in DMSO,1ml of alkaline DMSO (1ml DMSO containing, 5 mM NaOH in 0.1 ml water) was added to give a final volume of 1.4 ml. The absorbance was measured at 560 nm.[13]

RESULTS

The hydro alcoholic extract of leaves of *Lagerstroemia speciosa* showed presence of alkaloids, glycosides, carbohydrates, flavonoids, steroids, tannins, saponins, gums, mucilage, triterpenoids. (Table No. 1)

The total phenolic contents of hydro alcoholic extract of leaves of *Lagerstroemia speciosa* was expressed as gallic acid equivalent in mg/g of the extracts. The total phenolic contents of the extract is 20.18%.

Lagerstroemia speciosa hydro alcoholic extract was tested for its antioxidant activity in four different *in vitro* models. The antioxidant activity measured are given in Table No. 2. *Lagerstroemia* showed IC_{50} value 4.75±0.25 µg/ml for DPPH method, which was comparable to that of ascorbic acid (IC_{50}=2.25±0.29). For Hydrogen peroxide method, IC_{50} value was found to be 28.00±0.16 µg/ml, which compares favourable with Ascorbic acid (IC_{50}=187.33±3.45µg/ml). In Nitric oxide model, IC_{50} value was for to be 750.25±3.66 µg/ml, which is very low when compared to Rutin (IC_{50}=68.52±2.27mg/ml). The extract failed to exhibit antioxidant activity in the scavenging of superoxide radical by alkaline DMSO method. The IC_{50} values obtained, however, for the extract in all the methods were found to be higher than the standard used, indicating its low activity compared to the standards.

DISCUSSION

Free radicals have aroused significant interest among scientists in the past decade. Their broad range of effects in biological systems has drawn the attention of many experimental works. It has been proved that these mechanisms may be important in the pathogenesis of certain diseases and ageing. There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases.[14] Many synthetic antioxidant components have shown toxic and/or mutagenic effects, which have shifted the attention towards the naturally occurring antioxidants. Numerous plant constituents have proven to show free radical scavenging or antioxidants activity.[15]

Table 2: Antioxidant activity of leaves of *Lagerstroemia speciosa* (L) hydro alcoholic extract

Extract/Standard	IC_{50}+ values ± SEM* (µg/ml)			
	DPPH	H_2O_2	Nitric oxide	TBARS
Hydro alcoholic Extract	4.75±0.25	28.00±0.16	750.25±3.66	>1000
Ascorbic acid	2.75±0.29	187.33±3.45	-	>1000
Rutin	7.89±0.51	35.26±0.166	68.52±2.27	–

** Average of three independent determinations, three replicates, values are mean ± SEM.*

+ IC_{50} = Concentration of the sample/standard required to inhibit 50% of free radicals.

SEM = Standard error mean

H_2O_2 = Hydrogen peroxide

DPPH = 1, 1-diphenyl, 2-picryl hydrazyl

TBARS = Thiobarbituric acid reactive species

Flavonoids and other phenolic compounds of plant origin have been reported as scavengers.[16]

The preliminary phytochemical investigation of the extract revealed the presence of steroids, terpenoids, alkaloids, carbohydrate and phenolic compounds such as tannins, flavonoid etc., The phenolic compounds may contribute directly to anti oxidative action.[17] This result indicates that polyphenol present in leaves and its extract could be partly responsible for the beneficial effects.

In our present study demonstrated that, DPPH is a free radical, stable at room temperature, which produces a purple colour solution in methanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncoloured methanol solutions. Hydrogen peroxide radicals Scavenging activity showed that the extract is a potent scavenger of hydrogen peroxide. This hydrogen peroxide is mainly produced by enzymatic reactions. The ability of plant extracts to scavenge $H_2O_2$is followed by decay in H_2O_2concentration. Nitric oxide radical inhibition study proved that the extract is a moderate scavenger of nitric oxide. This nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract inhibits nitrite formation by competing with oxygen to react with nitric oxide directly and also to inhibit its synthesis. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide[12]. From the nitric oxide test, rutin was used as a standard. In the PMS/NADH –NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture.

This study suggested that the *Lagerstroemia speciosa* L plant extract possess antioxidant activity, which might be helpful in preventing or slowing the progress of various oxidative stress related diseases. Further investigation on the isolation and identification of antioxidant component(s) in the plant may lead to chemical entities with potential for clinical use.

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Antioxidant Availabiltiy of Beheda (*Terminalia bellerica (Roxb.)*) in Relation to its Medicinal Uses

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ABSTRACT

Oxidative stress arises from an imbalance in the generation and metabolisms of ROS, with more of ROS being produced, than that is metabolized. Oxidative stress plays a significant pathological role in human diseases. However, the antioxidant supplements may be used to help the human body to reduce oxidative damage. Therefore the present study evaluated the antioxidant activity of *Terminalia bellerica* (Gertn.) Roxb. fruits. The study was designed to evaluate and compare the antioxidant activity, total phenolics, flavonoids content of aqueous (AETB) and ethanolic (EETB) extracts of fruits. The antioxidant activity was assessed by DPPH (1,1–diphenyl–1,2–picryl hydrazyl), ABTS (2,2-Azino-bis 3-ethyl benothiazoline-6-sulfonic acid diammonium salt), nitric oxide, superoxide and hydroxyl radical scavenging assay, FRAP (Ferric Reducing Antioxidant Power), reducing power and TAC (Total antioxidant capacity). AETB has shown higher antioxidant activity (% inhibition) as compared to EETB in nitric oxide, superoxide, ABTS(2,2-Azino-bis 3-ethyl benothiazoline-6-sulfonic acid diammonium salt) radical scavenging assay with IC_{50} values 41.42±1.23, 892.85±2.73, 23.74±2.0 in AETB and 93.16±1.93, 3496.50±5.21, 55.89±2.21 in EETB respectively. Like antioxidant activity the reducing power increases in a dose dependent manner showing higher absorbance at 700 nm for AETB i.e. 0.95±0.03 as compared to EETB i.e. 0.43±0.01 at 500µg/ml. The FRAP values were found for AETB 1.68±0.07, EETB 1.06±0.03 and TAC values were found for AETB 2.53±0.07 and EETB 2.90±0.01. The amounts of total phenolic (TPC) and flavonoid content (TFC) were also determined. The results suggest that TPC and TFC contribute significantly to the antioxidant activity of the *Terminalia bellerica* (Gertn.) Roxb. Fruits.

Keywords: Antioxidant, FRAP assay, DPPH assay, *Terminalia bellerica*, ABTS assay, Total phenolic content.

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INTRODUCTION

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by the body's normal use of oxygen such as respiration and some cell mediated immune functions. Naturally, there is a dynamic balance between the amount of free radicals generated in the body and antioxidants to quench and/or scavenge them and protect the body against their deleterious effects.[1] The ROS inducing superoxide anionic radical (O_2^-), hydrogen peroxide (O_2^{-2}) and hydroxyl radicals (·OH) are implemented in oxidative damage to various cellular macromolecules. Increasing number of evidence suggested that oxidative stress induced biochemical changes are crucial etiological factors in several chronic human disease such as diabetes mellitus, cancer, atherosclerosis, arthritis, inflammation and neurodegenerative disease.[2] There have been many studies undertaken on how to delay or prevent the onset of these diseases. The most likely and practical way to fight against degenerative diseases is to improve body antioxidant status, which could be achieved by higher consumption of vegetables and fruits. Foods from plant origin usually contain natural antioxidants that can scavenge free radicals. The antioxidants may mediate their effect by directly reacting with ROS, quenching them and / or chelating the catalytic metal ions. Several synthetic antioxidants, e.g. BHA and BHT are commercially available but they are suspected to cause or prompt negative health effects, and also show low solubility and moderate antioxidant activity. Natural antioxidants, especially

phenolic and flavonoids are safe and also bioactive. Therefore, in recent years, considerable attention has been directed towards identification of plants with antioxidant ability that may be used for human consumption.[3]

Terminalia bellerica Roxb. (family: Combretaceae), commonly known as belleric myrobalan and locally known as beheda, is an edible plant found throughout Central Asia.[4] Its fruit has been used in traditional medial system for anemia, asthma, cancer, colic, constipation, diarrhoea, dysuria, headache, hypertension, inflammations, and rheumatism.[5,6] It contains termilignan, thannilignan, 7-hydroxy-3′,4′-(methylenedioxy) flavone, anolignan B,[7] gallic acid, ellagic acid, ß-sitosterol,[8] arjungenin, belleric acid, bellericoside[9] and cannogenol 3-O-ß-D-galactopyranosyl-(1→4)-O-α-Lrhamnopyranoside.[10]

Terminalia bellerica is known to lower the lipid levels in hypercholesterolemic animals.[11] The ethanolic extract of *Terminalia bellerica* was found effective against several pathogens including *Bacillus subtilis, Proteus vulgaris, Salmonella typhimurium, Salmonella typhimurium, Escherichia coli, and Staphylococcus aureus*.[12] On the other hand, methanolic extract (75%) of Terminalia bellerica reduced the serum glucose level both in normal and alloxan-induced diabetic rats,[13] showing preventive effect against the myocardial necrosis in rats[14] water soluble fraction obtained from the defatted fruits of *Terminalia bellerica* caused hepatoprotection against CCl_4-induced hepatotoxicity.[15,16,17] reported that *Terminalia bellerica* lowers blood pressure (BP). In this study, we explored the *in vitro* antioxidant activity of the fruits of this plant.

MATERIAL AND METHODS

Chemicals

Trolox (6-hydroxy-2,5,7,S-tetramethylchromam-2-carb-oxylic acid), ABTS(2,2-Azino-bis 3-ethyl benothiazoline-6-sulfonic acid diammonium salt) was purchased from Sigma Chemical Co. Ltd USA. DPPH (1,1 – diphenyl – 1,2 – picryl hydrazyl), TPTZ(2,4,6,-tripyridy-s-triazine), potassium ferricyanide, trichloroacetic acid (TCA), $FeCl_3$, sodium nitroprusside, sulphanilamide, napthylethylenediamine dihydrochloride, TPTZ(2,4,6,-tripyridy-s-triazine), ascorbic acid, NBT (nitroblue tetrazolium), reduced NADH (nicotinamide adenine dinucleotide), PMS (phenazine methosulfate), sulphuric acid (H_2SO_4), ammonium molybdate, ammonium persulphate,ascorbic acid/ standard Vitamin C (Vit. C), quercetin and pyrocatechol was purchased from HiMedia, Mumbai. All other unlabelled chemicals and reagents were of analytical grade and used without further purification.

Plant material

The *Terminalia bellerica (Roxb.)* fruits were collected from Govali village, Kalyan, Thane district, Maharastra, India. The plant material was taxonomically identified by Blatter Herbarium St Xavier's College, Mumbai. A voucher specimen (No. T-1114 of S.C. Tavakari) has been preserved in a laboratory for further reference. The collected fruits were dried under shade and powdered with a mechanical grinder and stored in an air tight container. The dried powder material of the fruits was soaked in distilled water and ethanol for 10hrs, to get an aqueous (AETB) and ethanolic extract (EETB) after filtration through Whatman paper No. 42.

Preliminary phytochemical screening

Qualitative phytochemical analysis of AETB and EETB was carried out as follows: Phenolics: 2ml of filtrate + 2ml $FeCl_3$, blue precipitate indicated presence of phenolics. Saponins (frothing test): 0.5 ml filtrate +5ml distilled water); frothing persistence indicated presence of saponins. Alkaloids: 2ml of filtrate +1%HCl+Dragendroff reagent, orange precipate indicate the presence of alkaloids. Flavonoids; 5ml dilute ammonia was added to a portion of filtrate +concentrated sulphuric acid; yellow colour indicates presence of flavonoids. Steroids (Liebermann-Burchard reaction: 2ml filtrate +2ml acetic anhydride +concentrated sulphuric acid; green color indicates the presence of steroids. Terpenoids: 4ml of filtrate +concentrated sulphuric acid 3ml was added to form a layer; reddish brown colouration interface indicates the presence of terpenoids. Cardiac glycosides (Keller-Kinliani test): 2ml filtrate + 1ml of glacial acetic acid + $FeCl_3$ +concentrated H_2SO_4; brown colour indicates the presence of cardiac glycosides.[18]

Total phenolic content

The total phenolic content of different extracts was measured using colorimetric Folin –Ciocalteu method. The reaction mixture consisted 5ml of diluted sample to which 3 ml of distilled water and 0.5 ml Folin –Ciocalteu reagent was added. After 3minutes, add 2ml of 20% Na_2CO_3 solution and place the tubes in boiling water bath for one min, cooled and the absorbance was measured at 760 nm. Standard graph was prepared by using different concentration of pyrocatechol.[19]

Total flavonoid content

The flavonoid content of different extracts was measured using a modified colorimetric method. 0.5ml of sample was

mixed with 0.5 ml of 2% $AlCl_3$ and incubated for 10mins. and the absorbance was measured at 415 nm. The measurement was compared to a standard graph for quercetin.[20]

Antioxidant Activity
Determination of reducing power (Fe3+ - Fe2+ transformation ability)

The reducing power of a compound serves as significant indicator of its potential antioxidant activity. Increased absorbance of the reaction mixture indicates increased reducing power.

Various conc. of the extracts in 1ml of water were mixed with phosphate buffer (2.5 ml, 0.2 M pH 6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (2.5 ml, 10%) were added to the mixture, which was then centrifuged al 3000g for 10 min. upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and freshly prepared $FeCl_3$ solution (0.5ml, 0.1%). The absorbance was measured at 700nm.[21]

Super oxide anion scavenging activity

1ml of NBT solution (144µM in 100mM phosphate buffer, pH 7.4), 1ml of reduced NADH (677µM in 100mM phosphate buffer, pH 7.4) and 0.5 ml of sample extract was mixed and the reaction was started with adding 100µl of PMS solution (60µM PMS in 100mM phosphate buffer, pH 7.4). The reaction mixture was incubated at 25°C for 5 min, and the absorbance 560 was measured against blank.[22]

Insert using Microsoft formulae

$$\% \text{ scavenging} = \frac{A_{con} - A_{test}}{A_{con}} \times 100$$

Nitric oxide radical scavenging activity

Sodium nitroprusside (5mM, 1ml) in phosphate buffer saline (PBS) (0.1 M, 7.4 pH) was mixed with 3 ml of different conc. of the extract and incubated at 25°C for 150 min. 0.5 ml of the samples was mixed with 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). Measure the absorbance at 546 nm.[23]

Insert using Microsoft formulae

$$\% \text{ scavenging} = \frac{A_{con} - A_{test}}{A_{con}} \times 100$$

DPPH radical scavenging activity

The assay is based on the measurement of the scavenging ability of antioxidant towards the stable radical DPPH. DPPH radical react with suitable reagent, the electrons become paired off and the solution looses color stoichiometrically depending on number of electrons taken up.A volume of 2ml of sample was added to 2ml of phosphate buffer (0.02M, pH 6) and 2ml of 0.2mM DPPH in 95% ethanol. The mixture was shaken and left for 30 min. at R.T. and the absorbance was measured at 517 nm.[24]

The capability to scavenge the DPPH radical was calculated using following equation:

Insert using Microsoft formulae

$$\% \text{ scavenging} = \frac{A_{con} - A_{test}}{A_{con}} \times 100$$

ABTS radical scavenging assay

For ABTS assay, the method of Re[25] was adopted. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate/ ammonium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml $ABTS^{+}$ solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The ABTS.+ scavenging capacity of the extract was calculated as ABTS radical scavenging activity (%) = [(Abscontrol - Abssample)]/(Abscontrol)] × 100 where Abscontrol is the absorbance of ABTS radical + methanol; Abssample is the absorbance of ABTS radical + sample extract/standard.

FRAP assay

The ferric reducing ability was measured by Ferric Reducing Antioxidant Power (FRAP) assay. The stock solutions of 10mM TPTZ in 40 mM HCl, 20mM $FeCl_3.6H_2O$ and 0.3 M acetate buffer (pH 3.6) were prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml of acetate buffer. It was prepared freshly and warmed at 37°C. 900µl of FRAP reagent was mixed with 90 µl of distilled water and 30µl of sample solution. The reaction mixture was then incubated at 37°C for 30 min and absorbance was recorded at 595 nm. The concentration of $FeSO_4$ was in turn plotted against concentrations of the standard antioxidants (L-ascorbic acid and Trolox).[26]

Total antioxidant capacity

0.1ml of extract was combined in eppendorf tube with 1ml of reagent solution (0.6M sulphuric acid, 28mM

sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated in thermal block at 95°C for 90 minutes. After cooling to room temperature; the absorbance of the aqueous solution of each was measured at 695 nm against blank. [27]

Statistical analysis

Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance i.e. one way ANOVA and student't' test using GraphPad QuickCalcs.. The two-tailed P <0.05 the difference is considered to be statistically significant and if P <0.0001 the difference is considered to be extremely statistically significant The IC_{50} values were calculated from linear regression analysis.

RESULT AND DISCUSSION

Preliminary phytochemical screening

Preliminary phytochemical screening of AETB and EETB revealed the presence of various bioactive components like flavonoids, sterols, terpenoids and phenolics while negative for the rest of classes of compounds.The presence of flavonoids and tannins in Terminalia bellerica might be contributing in its cardiovascular effects.[28]

Total phenolic and flavonoid content

Total phenolic compounds are reported as pyrocatechol equivalents. The total phenolic contents of AETB and EETB were 10.92±0.05 and 10.32±0.09 pyrocatechol equivalent/g of sample, respectively. The P value < 0.0001, this difference is considered to be extremely statistically significant. The total flavonoid contents of AETB and EETB were 10.27±0.11 and 2.9±0.01 mg quercetin equivalent/g of sample. The P value < 0.0001, this difference is considered to be extremely statistically significant. AETB had higher total phenol and flavonoids contents than EETB. It has been reported that soft fruits and medicinal plants exhibited higher levels of flavonoids.[29] Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities.[30]

Antioxidant activity

Antioxidant activity of the extracts of varying concentrations ranging form 10- 10000 µg/ml was evaluated by various in vitro models. It was observed that the test compounds scavenged free radicals in concentration dependent manner in all the models.
The result of FRAP and TAC assay were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) and Ascorbic acid Equivalent Antioxidant Capacity as described. TEAC is the concentration of Trolox (µmol/L) required to give the same antioxidant capacity as 1% (w/v) test substance. AEAC is the same for Ascorbic acid.[31]

Determination of reducing power (Fe3+ – Fe2+ transformation ability)

In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700nm.[32] Increasing absorbance at 700 nm indicates an increase in reductive ability. Table 1 shows that the reducing powers of all the extracts and standards also increased with the increase of their concentrations. There was an extremely significant difference (p< 0.001) among the AETB and EETB in reducing power. The activity of AETB and EETB was not comparable with Vit. C and trolox (p< 0.05) there were significant differences between them.

Super oxide anion scavenging activity

Superoxides are produced from molecular oxygen due to oxidative enzymes[33] of body as well as via nonenzymatic

Table 1 Reducing power ((Fe3+ - Fe2+ transformation ability) of AETB, EETB, Vit C and Trolox

conc.(µg/ml)	Absorbance at 700nm			
	AEMP	EEMP	Vit C	Trolox
5	0.09±0.01	0.04±0.00	0.20±0.02	0.11±0.01
10	0.16±0.03	0.18±0.01	0.23±0.02	0.13± 0.01
50	0.20±0.05	0.20±0.01	0.37±0.01	0.16± 0.02
100	0.32±0.01	0.27±0.02	0.48±0.02	0.2±0.001
500	0.95±0.01	0.43±0.01	0.88±0.03	0.58±0.02

Values are means ± S.D. (n=4)

reaction such as autoxidation by catecholamines.[34] In the present study, superoxide radical reduces NBT to a blue colored formazan that is measured at 560 nm.[35] Table 2a shows the superoxide scavenging effect of AETB in comparison to EETB on the PMS/NADH-NBT system. The increase of percentage scavenging activity thus indicates the consumption of superoxide anion in the reaction mixture by the plant extracts. AETB had strong superoxide radical scavenging activity as compared to EETB as IC_{50} value of AETB (892.85±2.73 µg/ml) is less than EETB (4166.66±5.21 µg/ml). There were significant differences ($p < 0.05$) between AETB and EETB. The IC_{50} value of Vit C is 66.31± 3.14, whereas Trolox shows at 2857.14± 1.01 µg/ml. Lower the IC_{50} value of better is the scavenging ability of the sample.

Nitric oxide radical scavenging activity

The extract effectively reduced the generation of nitric oxide from sodium nitroprusside (Table 2a). In vitro inhibition of nitric oxide radical is a measure of antioxidant activity of plant drugs. Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent.[36] Both AETB and EETB decreased the amount of nitrite generated from the decomposition of sodium nitroprusside in vitro which may be due to the presence of antioxidant principles in the extract. The percentage scavenging activity increased with increasing concentration of the extract. Lower the IC_{50} value of better is the scavenging abitity of the sample. There were significant differences ($p < 0.05$) between AETB and EETB. The IC_{50} value of AETB and EETB was found to be 41.42±1.23, 89.28±2.17. However, Std Vit. C activity of was very more pronounced than that of our extracts (162±1.32µg/ml.)

DPPH radical scavenging activity

DPPH assay is one of the most widely used methods for screening of antioxidant activity of plant extracts.[37] DPPH is a stable, nitrogen-centered free radical which produces violet colour in ethanol solution. It was reduced to a yellow coloured product, diphenyl picryl hydrazine, with the addition of all fractions in a concentration-dependent manner. All the concentraction of AETB and EETB demonstrated H-donor activity. Lower the IC_{50} value of better is the scavenging ability of the sample. The IC_{50} values of AETB and EETB were 38.4±2.28µg/ml and 28.24±1.18µg/ml respectively. These activities are less than that of the Std Vit C and Trolox i.e. 10.40±1.43µg/ml and 17.42±1.21µg/ml respectively (Table 2b). There were significant difference ($p < 0.05$) between AETB and EETB.

ABTS radical scavenging activity

Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals.[38] The AETB and EETB were fast and effective scavengers of the ABTS radical and this activity was comparable to that of Vit. C. Higher concentrations of the extracts were more effective in quenching free radicals in the system. AETB has shown higher antioxidant activity (% inhibition) as compared to EETB in ABTS (2,2-Azino-bis 3-ethyl benothiazoline-6-sulfonic acid diammonium salt) radical scavenging assay with IC_{50} values 24.71±2.21 in AETB and 50.12±0.87 in EETB respectively (Table 2a). There was significant difference ($p< 0.05$) between AETB and EETB. These activities are less than that of the Std Vit C and Trolox i.e. 33.94±1.23µg/ml and 5.42±0.27µg/ml respectively.

Table -2a % Radical scavenging activity of AETB and EETB in *in vitro* Assays at various concentrations

conc.(µg/ml)	% scavenging activity			
	Superoxide anion radical		Nitric Oxide radical	
	AEMP	EEMP	AEMP	EEMP
10	0.00±0.01	0.19±0.16	25.19±0.71	18.42±0.78
50	0.00±0.71	15.13±1.28	57.73±1.08	26.31±0.91
100	4.76±1.78	25.17±1.00	30.76±0.91	52.63±2.78
500	21.42±1.01	33.56±2.16	27.12±2.01	52.61±0.46
1000	59.52 ±1.91	48.46±2.10	27.16±1.47	52.78±3.01
5000	59.04 ±0.21	61.53±1.10	27.18±0.71	52.63±2.01

Values are mean ± S.D (n=4)

Table -2b % Radical scavenging activity of AETB and EETB in *in vitro* Assays at various concentrations

conc.(µg/ml)	% scavenging activity			
	DPPH radical		ABTS radical	
	AEMP	EEMP	AEMP	EEMP
10	23.41±2.14	29.41±0.70	30.28±0.01	16.23±0.16
50	63.04±1.17	86.17±1.28	99.14±0.71	50.07±1.28
100	69.81±2.28	95.90±2.17	99.73±1.78	86.06±1.00
500	82.18±2.18	95.10±0.18	99.02±1.01	94.17±2.16
1000	89.27±2.74	95.49±1.7	99.08±1.91	94.13±2.10
5000	89.27±1.11	95.05±1.10	99.86±0.21	94.23±1.10

Values are mean ± S.D (n=4)

FRAP assay

In FRAP (Ferric reducing antioxidant power) assay the ability of plant extract to reduce ferric ions was determined. FRAP assay measures the changes in absorbance at 593 nm owing to the formation of blue colored Fe^{+2}- tripyridyltriiazine compound from the colourless oxidized Fe^{+3} form by the action of electron donating antioxidants.[39] The FRAP values of AETB is higher as compared to EETB (Table 3). The P value < 0.0001, this difference is considered to be extremely statistically significant. Since FRAP assay is easily reproducible and linearly related to molar concentration of the antioxidant present it can be reported that AETB and EETB may act as free radical scavenger, capable of transforming reactive free radical species into stable nonradical products.

Total antioxidant capacity

Total Antioxidant capacity of AETB and EETB is shown in Table 3. The phosphomolybdenum method was based on reduction of MO (VI) to MO (V) by the antioxidant compound and the formation of green phosphate/ MO (V) complex at acidic pH.[40] In this assay EETB was found to have higher activity, AETB showed lower activity. The P value < 0.0001, this difference is considered to be extremely statistically significant. The extracts demonstrated electron donating capacity and thus they may act as radical chain terminators, transformating reactive free radical species into stable non reactive products.[41]

Table 3: FRAP ?? of AEMP and EEMP

1% (w/v) extracts used	FRAP Values (µmol/L)	TAC Values (µmol/L)
AETB (AEAC)	1.68±0.02	2.53±0.01
EETB (TEAC)	1.06±0.03	2.90±0.01

Values are mean ± S.D (n=4)

CONCLUSION

The results of present study demonstrate that the radical scavenging of AETB, indicate that the use of *Terminalia bellerica (Roxb.)* as a medicinal plant for the treatment of hypertension and possessing properties like antispasmodic and bronchodilatory seems quite justified.

The percentage scavenging activity (with reference to IC_{50} value) in super oxide anion, nitric oxide, ABTS radical scavenging assays shows that AETB has better percentage scavenging activity as compared to EETB. AETB thus also give effective reducing power and FRAP values, But the TAC values AETB are lower as compared to EETB. The overall better antioxidant and free radical scavenging activities of AETB might be due to the presence higher amounts of phenolic and flavonoid compounds in aqueous extract.

Further studies are in progress in our laboratory to evaluate the in vivo antioxidant potential of this extract in various animal models and phytochemical studies are required to establish the types of compounds responsible for the bioactivity of this medicinal plant.

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Indian Medicinal Plants Used in Hair Care Cosmetics: A Short Review

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ABSTRACT

Plants act as a source of food and medicine from long times. A wide range of plant oils are used in cosmetics and toiletry preparations. Hair is an important part of body, reflect personality of person. There are many cosmetics available for hair care. From long time plant materials are used for hair care. In this review, Indian medicinal plants having hare care properties are summarized in terms of their biological source, active constituents and biological activity.

Keywords: hair care formulations, hair tonic, natural plants, hair cosmetics.

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INTRODUCTION

In olden times herbal products were used for medicinal purposes, both internally as well as externally. Herbal drugs were used as juice, latex or in dried powder form.[1] Now a day's personal care products containing ingredient from the plant origin are getting an increasing trend in the pharmacy world. Cosmetic product containing plant material as active ingredient is comes under the category of cosmeceuticals.[2–3] Appearance of hair makes an important impact on total body feature. Color, length and appearance of hair make a significant difference from person to person. Cosmetics that are used for hair care purpose applied orally and should not be used for therapeutic purpose.[4–5] Basic feature of hair care cosmetics are as-

1. Should be easy to use
2. Should have local effect.
3. Should be harmful to hair skin and mucous membrane.
4. Should not be allergic to body.
5. Should be applied topically.

Hair care cosmeceutical formulations mainly include shampoo, gel, lotion solution and oil.

Different herbs used in hair care

Amla (Emblica officinalis)

Amla (Emblica officinalis, Family- Euphorbiaceae) is deciduous tree widely found in India at the height of 350 m. It is often cultivated at commercial level in Uttar Pradesh, Gujarat, Rajasthan and Maharashtra. Amla contains 5-6% of tannins such as gallic acid, ellagic acid and phyllembelin. It is commercialized in the form of shampoos and hair oil. Mainly its oil and aqueous extract is used in the hair care formulations.[5]

Brahmi (Centella asiatica)

Brahmi (Centella asiatica, Family- Umbelliferae) is herbaceous creeping herb growing at the bank of river. In India, brahmi is found in wet damp and marshy places of north India. Brahmi contains essential oils, sterols, flavonol, glycoside and triterpenoid saponins. Brahmi oil and soft extract are two commercial preparation of brahmi used in hair care formulations. It is also categorized as rasayan in ayurveda and hence possesses the properties of delaying ageing signs in body like graying of hairs. Brahmi also helps in relieving mental fatigue and hence helps in maintain proper bodily environment that leads to healthy hairs.[6]

Bhringraj (Eclipta alba Linn.)

Bhringraj (Eclipta alba Linn, Family- Asteraceae) is an annual or perennial plant found in moist places throughout India, ascending up to 600 fts. Bhringraj mainly contains coumestans (wedelolactone and dimethyl wedelolactone), alkaloid (ecliptine), glycosides (β-amyrin), triterpenic acid and steroids (ecalbasaponins). Brahmi oil is very good hair tonic and used as a constituent in hair formulation for healthy, black and long hair.[5–7]

Coconut (Cocos nucifera Linn.)

Coconut (Cocos nucifera Linn, Family-Palmae) tree is tall rising to a height of 30 meters, grow near the sea side. Oil of coconut fruit is used in different hair formulations such as shampoos and hair oil. Coconut oil has good saponification value so used in shampoos for hair care.[7]

Eucalyptus (Eucalyptus globulus)

Eucalyptus (Eucalyptus globulus, Family- Myrtaceae) is a long tree, cultivated in India for production of oil. Oil contains mainly cineole and lesser amount of volatile aldehyde, terpenes, alcohol and phenol. The oil is colorless or pale yellow liquid that has a characteristics aromatic odor and a pungent, spicy and cooling taste. It produces very satisfactory results in scruff and chafes dandruff.[6–7]

Henna (Lawsonia inermis)

Henna (Lawsonia inermis, Family- Lythraceae) grows wild and cultivated as garden plant throughout India. Henna leaves are the part of plant that is used in hair formulations. Leaves mainly contain lawsone (quinone) dissolve in basic pH to give dark intense orange color. Henna leaves have been used from ancient time as a hair colour due to the chemical interaction of lawsone (thiol group) to the keratin.[6, 7, 8, 9]

Neem (Azadirachta indica)

Neem (Azadirachta indica, Family- Melliaceae) is indigenous to all plains in Indian subcontinent. It also grows widely in the sub-Himalayan track at altitude of 700–10, 000 m above sea level. Neem leaves contain flavonoids, steroids, terpenoids, sterols and nimbolide. Neem seed and seed oil contain different bitter limonoids including nimbin, nimbibin, salanin etc. many commercial shampoos contain neem oil for the control of ticks, fleas and lice. In European countries neem oil also used in different herbal hair oil, hair tonic and conditioners.[6–7]

Gurhal (Hibiscus rosa sinensis)

Gurhal (Hibiscus rosa sinensis, Family-Malvaceae) is a popular Indian garden plant. Gurhal contain taraxeryl acetate, beta-sitosterol, campesterol, stigmasterol, cholesterol, erogosterol, flavonoids and flavonoid, glycosides, lipids, citric and oxalic acids. In herbal formulations, hibiscus petal is used to stimulate thicker hair growth and to prevent premature graying, hair loss and scalp disorders. Petals extract acts as a natural hair conditioner and can be used in hair washes.[6–10]

Jatamansi (Nardostachys jatamansi)

Jatamansi (Nardostachys jatamansi, Family-Valerianaceae) is grown widely in India, especially north India to east India. These are found in the alpine Himalayas at an altitude of 3000–5000. Rhizomes of jatamansi contains 1 to 2 % of pale yellow volatile oil, jatamansic acid, and ketones (jatamansone and nardostachone), resin, sugar etc. oil obtained from rhizomes of this plant used in hair tonic preparations, to promote the growth of hair and impart blackness. Jatamansi is a useful hair tonic and is commonly used in hair oils, promoting hair growth and luster. It promotes hair growth and imparts black color to the hair.[7]

Fenugreek (Trigonella foenum graecum)

Fenugreek (Trigonella foenum graecum, Family-Leguminoseae) plant is a quick growing annual leguminous herb about 2 feet in height. In India Fenugreek (Trigonella foenum graecum, Family- Leguminoseae) is often cultivated as a cover crop in citrus-fruit groves to take advantage of their leguminous nature. The major producers of Indian fenugreek are Rajasthan, Gujarat, Uttar Pradesh and Tamilnadu. Generally seed is used to prepare hair care formulations. Seed contain alkaloids (Neurin, Trigonelline, Choline, Gentianine), amino acids (Isoleucine, 4-Hydroxyisoleucine, Histidine, Leucine, lysine), Saponins (Graecunins, fenugrin B, fenugreekine, trigofoenosides A-G), lipids, vitamins and fibers. Traditionally fresh Fenugreek leaves paste applied over the scalp regularly before bath helps hair grow, preserves natural color, keeps hair silky and also cures dandruff.[6, 10, 11]

Cedar wood oil (Juniperus virginiana)

Cedar wood oil extracted from the woods (Juniperus virginiana, Family-) for hair loss and dandruff. The chief components of cedar wood essential oil are alpha cedrene, beta cedrene, cedrol, widdrol, thujopsene and a group of sesquiterpenes, which contribute to its

medicinal values. Cedarwood oil is used for hair care formulations, especially for dry hair, and to induce hair growth along with other essential oils. Even people having the problem of dandruff can too benefit from using cedarwood oil.[9, 11, 12]

Rosemary oil (Rosmarinus officinale)

Rosemary (Rosmarinus officinale Linn, Family- Labiatae) is cultivated in Indian gardens. It contains volatile oil, resin, ursolic acid etc. Commercially rosemary oil is used in hair lotions and hair gels to promote hair growth and shining.[6]

Shikakai (Acacia concinna)

Shikakai (Acacia concinna, Family-Mimosaceae) is a shrub widely found in plains of central and south India. Shikakai literally means fruit of hairs. It is an excellent natural hair cleanser and astringent and also acts as detangle. It is used in soaps and shampoos for hair wash, promotes hair growth, removes dandruff and strengthens hair.[5–7]

Almond oil (Prunus dulcis)

Almond oil (Prunus dulcis, Family- Rosaceae) is obtained from ripe seeds of Prunus dulcis by cold expression technique. It is commerciaaly cultivated in north part of India. Almond oil contains 40-55% fixed oil, about 20% proteins, mucilage and emulsion. The fixed oil constituents are mainly oleic acid, linoleic acid, and palmitic acid. It is commercially used in dermatology as hair lotion and hair tonic.[11]

Ginko (Ginko biloba)

Ginko (Ginko biloba, Family- Ginkgoaceae) are obtained as dried leaves from Ginkgo biloba. It is commercially grown in North India. The levees chiefly contain terpenes, ginkolides, flavanol glycosides and kaemferol. It is commercialized in the form of shampoos and hair lotions. Mainly leaf extract is used in the hair care formulations.[10–11]

Sandalwood oil (Santalum album)

Sandalwood oil (Santalum album, Family- Santalaceae) is from the heartwood of obtained from Santalum album an evergreen tree 8-12 meter in height by steam distillation method. It is indigenous to South India, and grows in the Western Ghats and a few other mountains. The Sandalwood oil contains 90-97% of sesquiterpenes alcohol (Santalol), hydrocarbons, acids, aldehydes and ketones. Sandalwood oil is employed as hair care products.[9–11]

Sesame oil (Sesamum indicum)

Sesame oil (Sesamum indicum, Family- Pedaliaceae) is obtained from ripe seeds of Sesamum indicum, an annual herb by expression technique. Sesame is produced commercially in Gujarat, West Bengal, Rajasthan, Tamilnadu, Orissa, Madhya Pradesh, Andhra Pradesh, Maharashtra, Uttar Pradesh, Punjab, and Karnataka in India. Sesame oil contains palmitic acid, oleic acid, linoleic acid, linolenic acid, stearic acid, arachidic acid, behenic acid, and gandoleic acid. Sesame oil is used commercially for hair tonic formulations.[8]

Senna (Cassia angustifolia/Cassia acutifolia)

Senna (Cassia angustifolia/Cassia acutifolia, Family-Leguminoceae/Fabaceae) is obtained as senna leaf from dried leaflets of Cassia angustifolia (Indian senna) & Cassia acutifolia (Alexendrian senna). The senna (Cassia angusifolia) is most commercially obtained from south india and some from the north part of India. The senna leaves contain rhein, chrysophanol, emodin, aloe emodin, mono and diglucosides, kaemferol. Palmidin, myricyl alcohol and mucilage. The leaves are commercially employed as hair black dye.[7–9]

Lemon oil (Citrus limonum)

Lemon oil (Citrus limonum, Family-Rutaceae) is obtained from peels Citrus limonum by cold extraction technique. The lemon is commercially grown in north-west region of India. The lemon oil contains α-pinene, camphene, β-pinene, sabinene, myrcene, α-terpinene, linalool, β-bisabolene, limonene, trans-α-bergamotene, nerol and neral. Lemon oil is used as hair cleaning agent.[9–12]

Rose Oil (Rosa Damascena)

Rose oil (Rosa Damascena, Family- Rosaceae) is obtained from fresh petals of Rosa Damascena by hydro-steam distillation technique. Rose is grown in almost all the parts of India. Rose oil contains citronellol, geranoil, linalool, farnesol, stearoptene, camphene, eugenol and pienene. Rose oil is employed as Hair care products.[3, 7, 11]

Sage oil (Salvia officinalis Linn)

Sage oil (Salvia officinalis Linn, Family-Labiatae) is obtained from dried leaves of Salvia officinalis Linn by steam distillation technique. The Sage is shrubby perennial plant cultivated in India. The Sage oil contains α-pinene, cineole, linalyl acetate, thujone (44 to 45%), borneol, bornyl acetate, farnesol, and camphor. The sage oil is employed as anti dandruff agent.[5–13]

Basil Oil (Ocimum Sanctum)

The Basil oil (Ocimum Sanctum, Family- Labiatae) is obtained from leaves & flowering tops of Ocimum Sanctum by steam distillation method. Basil Sanctum is cultivated in India widely. The basil oil contain 1, 8 cineol, linalool, citral, methyl chavicol (estragole), eugenol and methyl cinnamate. Basil oil stimulates and promotes hair.[7–13]

Jojoba oil (Simmondsia chinensis)

Jojoba oil (Simmondsia chinensis, Family-Simmondiaceae) is obtained from seeds of Simmondsia chinensis by cold pressed method. The Jojoba plant is mainly grown in Rajasthan, Gujarat, Maharashtra, and Tamilnadu in India. The Jojoba oil contain Eicosenoic acid, Docosenoic acid and oleic acid. Jojabo oil is used as a revitalizing agent for hair.[13]

Neem oil (Azadirachta indica)

Neem oil (Azadirachta indica, Family- Meliaceae) is obtained from powdered seeds, kernels or leaves of Azadirachta indica. Neem is native of India and grows throughout all parts of India. Neem oil contains Linoleic Acid, Lower Fatty Acids, Palmitic Acid, Stearic Acid and Oleic Acid. Neem oil is used as hair tonic and anti dandruff agent.[2, 5, 13]

Methi (Trigonella foenum-graecum L)

Methi (Trigonella foenum-graecum L, Family- Fabaceae) is obtained from dried ripe seeds of Trigonella foenum-graecum L. Methi plant is cultivated mainly in Rajasthan, Gujarat, Uttar Pradesh, Tamil Nadu and Madhya Pradesh in India. Methi contain essential amino acids, protein, starch, sugars, mucilage, mineral matters, volatile oil, fixed oil, vitamins and enzymes. Methi is used as hair care agent.[13–14]

Arnica (Arnica Montana)

Arnica (Arnica Montana, Family-Apiaceae) is obtained from dried roots and flowers of Arnica Montana. Arnica is a perennial herb cultivated mainly in north region of India. Arnica contains Arnicin, volatile oil, Tannin and phulin. Arnica is used for hair tonics and anti-dandruff preparations.[14]

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