Investigating the chemical Composition and the antimicrobial activity of the essential oil and Crude Extracts of *Sedum Microcarpum* (Sm.) schönl Growing Wild in Jordan

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ABSTRACT

Background: Several *Sedum* species, such as *S. album* L., S. acre L., *S. palaestinum* Bieb, and *S. pallidum* are used in traditional medicine to heal wounds, hemorrhoids, constipation, food fungi, and as laxative and diuretic agents. **Materials and Methods:** In this study, the antimicrobial activities of the hexane, n-butanol and aqueous methanol extracts as well as the essential oil from Jordanian *sedum microcarpum* (Sm.) schönl were investigated and Analysis of the oil constituents by using Gas chromatography-Mass spectrometer (GC/MS). **Conclusion:** The hexane extract showed no antibacterial activity against seven strains. The butanol and aqueous methanol extracts were active against the Gram-positive bacteria *Staphylococcus epidermidis*, *Bacillus cereus* and the Gram negative bacteria including *Enterococcus faecalis*, *Escherichia coli, Klebsiella pneumonia* and *Serratia marcescens*. Phytochemical screening of the active extracts revealed the presence of a mixture of quinones, tannins, alkaloids and flavonoids. The essential oil showed moderate activities against the tested strains. Analysis of the oil using Gas chromatography-Mass spectrometer (GC/MS) revealed a complex mixture of aldehydes, mono- and sesquiterpenes in addition to alkaloids. Oxygenated monoterpenes dominated the different classes of compounds detected (36.39%). The major volatile constituents from *S. microcarpum* included Myrtenol (21.7%), Caryophyllene oxide (8.2%) and z-1, 6-dioldihydromyrcene (5.87%).

Keywords: Antibacterial activity, Crassulaceae, Essential oil, GC-MS, Phytochemical screening, *Sedum microcarpum*.

INTRODUCTION

Sedum L. is one of the largest genera in the family Crassulaceae, comprising about 500 species distributed mainly in the Mediterranean region. The genus includes annual succulent herbs and shrubs inhabiting mostly open, sunny and arid habitats, and in some cases moist habitats. They are distinctive in having acuminate fleshy leaves, fleshy stems and yellow, yellowish or creamy to white tiny flowers.[1,2] Several *Sedum* species, such as *S. album* L., S. acre L., *S. palaestinum* Bieb, and *S. pallidum* are used in traditional medicine to heal wounds, hemorrhoids, constipation, food fungi, and as laxative and diuretic agents.[3–6]

Previous phytochemical screening of in the genus *Sedum* have shown that the genus is an excellent source of a variety of secondary metabolites including condensed tannins, alkaloids, flavonoids, hydroquinone, arbutin, free sugars, sedoheptose, wax esters, cyanogenic compounds, and triterpenoids.[7–16] However, little is known about the volatile constituents of *Sedum* and their biological activities and, to our knowledge, only two species

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(*Sedum pallidum* var. *bithynicum* and *S. spurium*) growing wild in Turkey have so far been examined for the chemical composition of their essential oils.[6] It has been found that the oil from *S. pallidum* was dominated by caryophyllene oxide (12.8%), *n*-nonanal (9.4%), α-bisabolol (6.8%), β-sesquiphellandrene (4.5%), and β-bisabolene (3.7%), whereas that from *S. spurium* was dominated by hexahydrofarnesyl acetone (15.7%), (*Z*)-phytol (10.2%), δ-cadinene (4.7%), *allo*aromadendrene (3.8%), and geranyl acetone (3.6%).[6] In Jordan, the genus is represented by nine naturally-occurring species. These include *S. laconicum* Boiss. and Heldr., *S. nicaeense* All., *S. microcarpum* (Sm.) Schönl., *S. caespitosum* Cav., *S. hispanicum* L., *S. rubens* L., *S. Pallidum* Bieb., *S. litoreum,* and *S. palaestinum* Boiss.[1]

Sedum microcarpum (Telmissa microcarpa; small-fruited stonecrop) (Sm.) Schönl. is a species of basically the Mediterranean Woodlands and Shrublands, Semi-steppe shrublands, and Shrub-steppes. This species is fairly abundant in the North West part of Jordan and extending, although rarely, to central regions.[1] It is an annual succulent herb growing largely in moist shallow cavities of rock outcrops, and distinguished by its showy red succulent leaves and branches, tiny white flowers, and one-seeded non-dehiscent nut-like fruit.[1,17] In this study, the chemical composition of the hydro-distilled essential oil of *S. microcarpum* was analyzed using GC/MS analysis techniques. Moreover, phytochemical screening of the crude extracts was performed to determine the identity of the major secondary metabolites. The antibacterial activities of these extracts were also evaluated.

MATERIALS AND METHODS

Plant material and fractionations

Whole plants of *S. microcarpum* were collected on February 2010 from natural populations around the city of Irbid, North of Jordan. The plant was identified using regional floristic literature.[18]

Sample treatment

The ground, whole air dried plant material (1.5 kg) was defatted by soaking in petroleum ether at room temperature (7 days) and then repeatedly soaked in ethanol at room temperature (50 L, 5 times, 7 days each). The residue obtained upon removal of ethanol under reduced pressure (107 g) was partitioned between chloroform and water. The dried chloroform layer was further partitioned between 10% aqueous methanol and hexane furnishing the aqueous methanol and hexane extracts. The polar organic compounds were extracted from water using n-butanol.

Isolation of the essential oil

Three fresh *S. microcarpum* samples (600 g powder) were subjected to hydro-distillations for 3.0 h using a Clevenger-type apparatus, yielding *ca.* 0.067% (w/w), pale yellow fragrant oils. Oils were then separately dried over anhydrous sodium sulfate and immediately stored in hexane at 4°C until use for further analysis by gas chromatography/mass spectrometry (GC/MS).

Qualitative analysis

The presence of the secondary metabolites of the tannins, flavonoids, anthocyanins, saponins, coumarines, quinones, anthraquinones and alkaloids was examined according to the methods of Kokate[19] and Harborne.[20] Detection of these phytochemicals was based on the visual observation of color change or formation of a precipitate after the addition of specific reagents. Table 1 summarizes the data of the various tests performed on the butanol and aqueous methanol extracts of *S. microcarpum* from Jordanian origin.

GC-FID analysis

Quantitative analysis of the essential oil was carried out using a Hewlett Packard HP-8590 gas chromatograph equipped with a split-splitless injector (split ratio, 1:50) and an FID detector. An OPTIMA-5 fused silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness) was used. The oil was analyzed under a linear temperature program applied at 3°C/min starting from 60°C through 246°C. Temperatures of the injector and detector were maintained at 250°C and 300°C, respectively. Concentrations of oil ingredients were determined using their relative area percentages obtained from GC chromatogram.

GC-MS analysis

The chemical analysis of the essential oil was carried out using GC-MS (Varian chrompack CP-3800 GC/MS/MS-200 (Saturn, Netherlands). The chromatographic conditions were as follows: column oven program,

Table 1. Phytochemicals detected in extracts of *S. microcarpum.*

Compounds groups	Butanol	Aqueous methanol
Quinones	+	+
Anthocyanins	–	–
Anthraquinones	–	–
Flavonoids	+	+
Tannins	+	+
Alkaloids	+	+
Saponins	–	–
Coumarins	–	–

Key: (+) major class detected; (–): Class not detected

60ºC (1 min, isothermal) to 246ºC (3 min, isothermal) at 3ºC/min; the injector and detector temperatures were 250 and 300ºC, respectively. Helium was the carrier gas (flow rate 0.90 mL/min). An HP-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thicknesses) was used. The actual temperatures in MS source reached 180ºC and the ionization voltage was 70 eV. A hydrocarbon mixture of *n*-alkanes (C_8 – C_{20}) was analyzed separately by GC/MS under same chromatographic conditions using the same HP-5 column.

Compound identification

Experimental retention indices of all the components were determined by Kovats method[21] using *n*-alkane (C_8 – C_{20}) mixture as a standard. Compound identification was achieved by comparing the experimentally obtained RI with those listed in literature,[22] co-injection of individual authentic components including pinenes (α and β), linalool, borneol, *n*-nonane, *n*-decanal, *n*-tetradecane, *n*-pentadecane, *n*-heptadecane, *n*-nanodecane, and by matching the mass spectral data with those held in the National Institute of Standards and Technology (NIST) and Wiley libraries of mass spectra and literature comparison.[22–24]

Antimicrobial study

In vitro antimicrobial activity of essential oil and hexane, butanol and aqueous methanol fractions from *S. microcarpum* were examined against seven different bacterial isolates (obtained from the Department of Biological Sciences, Yarmouk University, Jordan) using two different methods, namely the agar disc diffusion and the agar well diffusion methods. The seven bacterial isolates investigated included three *Gram*-positive bacteria *Bacillus cereus* (ATCC 11778), *Staphylococcus epidermidis* (ATCC 12228), and *Enterococcus faecalis* (ATCC 29212) and four *Gram*-negative bacteria *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 10031), *Serratia marcescens* (ATCC 27117) and *Proteus vulgaris* (ATCC 29905). Bacterial strains were cultured overnight at 37ºC in Trypton Soy broth (TSA).

Antibacterial activity was tested according to Parekh *et al.* (2005) and Vuddhakul *et al.* (2007)[25,26] with a slight modification. The molten Mueller Hinton agar was inoculated with the inoculums (1×10^8 CFU/ml) and poured into Petri plates. For agar disc diffusion method, the disc (0.7 cm) was saturated with 50 μg/mL of the test extract, dried, and introduced on the upper layer of the seeded agar plate. For agar well diffusion method, six equidistant wells (7 mm in diameter) were cut from the agar using a cork-borer. The test extract (50 μg/mL) was introduced into the well. The plates were incubated overnight at 37ºC. Microbial growth was determined by measuring the diameter of the inhibition zone. For each bacterial strain, controls were maintained and pure solvents were used. At the end of the period, all plates were examined for any zones of growth inhibition and the diameters of these zones were measured in millimeters. All tests were performed in duplicate and the results were shown as mean values.

A dilution agar method was used to determine the minimum inhibitory concentrations (MIC). Stock solutions were obtained by dissolving extracts in dimethylsulfoxide (DMSO 1%). Serial dilutions were made to obtain concentrations ranging from 0 to 1000 μg mL^{-1} for each of the essential oil, hexane, butanol and aqueous methanol fractions. Each mixture was added to Mueller–Hinton agar.[27,28] The Petri dishes contained a sterile solution of DMSO and the culture medium, respectively, after incubation at 37ºC for 24 h. The experiments were performed in triplicate.

RESULTS AND DISCUSSION

Table 1 shows the presence of various types of secondary metabolites in *S. microcarpum*, including quinones, flavonoids, tannins and alkaloids. Anthocyanins, anthraquinones, saponins, and coumarins were not detected in both the butanol and aqueous methanol fractions. Hydrodistillation of the fresh plant material yielded 40.0 mg of pale yellow oil, with an output of 0.067% (w/w). The general chemical profile of the essential oil, the percentage content, the retention indices of the constituents and the chemical class distributions of the essential oil components of the plant are summarized in Table 2. The compounds were separated into nine classes and were dominated by oxygenated monoterpenoids (36.39%). The remaining classes included aliphatic monoterpenes (1.64%), sesquiterpenes (8.70%), sesquiterpenoids (19.80%), alkaloids (2.18%), aldheydes (8.94%), alcohols (6.87%), alkanes (10.25%), and others (2.41%). The major compounds found in the essential oil of *S. microcarpum* were Myrtenol (21.7%), Caryophyllene oxide (8.2%), (Z)-1,6-diol dihydromyrcene (5.87%) decanol (4.91%), n-heptadecane (4.59%) and n-decanal(4.44%). Yayli et al. (2010) examined the constituents of essential oil from *S. pallidum* var. *bithynicum* and *S. spurium*. They found that the oil from *S. pallidum* was dominated by caryophyllene oxide (12.8%), *n*-nonanal (9.4%), α-bisabolol (6.8%), β-sesquiphellandrene (4.5%), and β-bisabolene (3.7%), whereas that from *S. spurium* was dominated by hexahydrofarnesyl acetone (15.7%), (Z)-phytol (10.2%), δ-cadinene (4.7%), *allo*aromadendrene (3.8%), and geranyl acetone (3.6%).[6] In this study, components of the essential oil from *S. microcarpum* were slightly different (Table 2). Results

Table 2. Identified components in the essential oils of *S. microcarpum* from Jordan, (values given are average of three independent measurements).

No.	RI[b]	Compound[a]	% Area	Identification method[c]
1-	804	Hexanal	1.25	MS, RI
2-	836	Furfural	0.39	MS, RI, Co
3-	861	3z-Hexenol	0.73	MS, RI
4-	907	n-Nonane	0.53	MS, RI, Co
5-	941	α-Pinene	0.45	MS, RI, Co
6-	981	β-Pinene	1.19	MS, RI,
7-	997	2-Pentyl furan	0.16	MS, RI,
8-	1073	*trans*-Linalool oxide	0.18	MS, RI,
9-	1090	*cis*- Linalool oxide	1.29	MS, RI,
10-	1103	Linalool	0.15	MS, RI, Co
11-	1108	n-Nonanal	1.43	MS, RI, Co
12-	1134	Terpineol	1.94	MS, RI,
13-	1150	Myrcenone	0.71	MS, RI,
14-	1202	Myrtenol	21.7	MS, RI, Co
15-	1204	n-Decanal	4.44	MS, RI,
16-	1208	Borneol	0.46	MS, RI, Co
17-	1231	Methyl -3,6-octadecanoate	0.11	MS, RI,
18-	1236	Nerol	0.21	MS, RI,
19-	1254	Geraniol	1.34	MS, RI,
20-	1265	Linalyl acetate	1.15	MS, RI,
21-	1274	Decanol	4.91	MS, RI,
22-	1298	n-pentyl pyrole	2.18	MS, RI,
23-	1305	4α-methyl-1,2,3,4α,5,6,7-octahydronaphthalene	0.40	MS, RI,
24-	1316	*p*-Guaiacol	1.43	MS, RI,
25-	1352	Thymol acetate	1.20	MS, RI,
26-	1360	Eugenol	0.61	MS, RI,
27-	1392	Vanillin	0.58	MS, RI, Co
28-	1397	n-Tetradecane[b]	1.83	MS, RI,
29-	1443	*trans*-β-5,6-epoxide ionone	3.44	MS, RI,
30-	1460	Alloaromadendrene	2.95	MS, RI,
31-	1462	Dehydroaromadendrane	1.52	MS, RI,
32-	1487	Germacrene D	0.12	MS, RI,
33-	1500	n-Pentadecane	0.36	MS, RI,
34-	1517	Menthyl pentanoate	0.19	MS, RI,
35-	1541	α-Calacorene	0.67	MS, RI,
36-	1558	Elemicin	0.10	MS, RI,
37-	1563	Ledol	0.40	MS, RI,
38-	1578	Caryophyllene oxide	8.20	MS, RI,
39-	1600	Pentadecanal	0.85	MS, RI,
40-	1619	epi-Cedrol	1.91	MS, RI,
41-	1622	Z-1, 6-diol-Dihydromyrcene	5.87	MS, RI,
42-	1664	Dihydro Eudesmol	0.38	MS, RI,
43-	1674	Caryophyllenol-II	2.58	MS, RI,
44-	1680	Khusinol	2.43	MS, RI,
45-	1687	α-Bisabalol	0.90	MS, RI,
46-	1687	8-cerden-13-ol	0.18	MS, RI,
47-	1694	*tans*-α-Bergamotal	0.14	MS, RI,
48-	1700	n-Heptadecane	4.59	MS, RI, Co
49-	1718	Curcuman-15-ol	0.13	MS, RI,
50-	1718	2Z,6Z-Farnesol	0.17	MS, RI,
51-	1772	n-Pentadecanol	1.23	MS, RI,
52-	1820	Acorone	0.25	MS, RI,
53-	1843	6,10,14-trimethyl-2-Pentadecanone	1.76	MS, RI,
54-	1900	n-Nanodecane	2.54	MS, RI, Co
55-	1916	Farnesyl acetone	0.37	MS, RI,

(Continued)

No.	RI[b]	Compound[a]	% Area	Identification method[c]
		Total identified	97.18	
		Monoterpenes hydrocarbon	1.64	
		Oxygenated monoterpenoids	36.39	
		Sesquiterpene hydrocarbons	8.70	
		Oxygenated Sesquiterpenoids	19.80	
		Alkaloids	2.18	
		Aldehydes	8.94	
		Alcohols	6.87	
		Alkanes	10.25	
		Others	2.41	

[a]Compounds are listed in order of their elution from a HP-5 column. [b]RI, retention index on HP-5MS column, experimentally determined using homologous series of C_8–C_{20} alkanes. [c]Identification methods: MS, by comparison of the mass spectrum with those of the computer mass library Wiely7Nist; RI, by comparison of RI with those reported in literature; Co, co-injection with authentic compound.

indicate that the essential oil of *S. microcarpum* shares some different components with other species of *Sedum* (*S. pallidum* var. *bithynicum* and *S. spurium*) which can be explained by the environmental factors.

The antimicrobial activities of the hexane, butanol and aqueous methanol fractions of *S. microcarpum* were assayed in vitro by the agar dilution method[25,26] against seven bacterial strains. The bacterial species were chosen as representatives of two major groups of microorganisms: *Gram*-positive bacteria and *Gram*-negative bacteria. The results are presented in Tables 3 and 4. No inhibitory effects of the hexane fraction extract against the seven bacterial strains were found. However, moderate antibacterial activity was shown by butanol and aqueous methanol extracts against *Staphylococcus epidermidis, Enterococcus faecalis*, *Escherichia coli, Klebsiella pneumoniae* and *Serratia marcescens*, but no activity against *Proteus vulgaris* and *Bacillus cereus* was observed. The antibacterial activity of the essential oil from *S. microcarpum* was also evaluated on the seven bacterial species. Moderate activities against all tested organisms compared to

Table 3. Antimicrobial activity of extracts and essential oil from *S. microcarpum*.

Microorganisms	Inhibition Zone (mm)					
	Hexane extract 1000 µg/mL	Butanol extract 1000 µg/mL	Aq-MeOH extract 1000 µg/mL	Essential oil 1000 µg/mL	Amoxicillin 100 µg/mL	Cephalexin 100 µg/mL
E. coli (ATCC 25922)	–	12	10	14	32	35
S. marcescens (ATCC 27117)	–	10	8	12	20	22
K. pneumoniae (ATCC 10031)	–	10	9	17	18	21
P. vulgaris (ATCC 29905)	–	–	–	10	18	23
B. cereus (ATCC 11778)	–	–	–	23	22	29
E. faecalis (ATCC 29212)	–	11	19	–	24	33
S. epidermidis (ATCC 12228)	–	13	21	26	30	35

Table 4. Minimal inhibitory concentration (MIC) (µg mL^{-1}) of extracts and essential oil from *S. microcarpum*.

Microorganisms	(MIC) (µg mL^{-1})			
	Hexane extract	Butanol extract	Aq-MeOH extract	Essential oil
E. coli (ATCC 25922)	–	625	650	500
S. marcescens (ATCC 27117)	–	700	750	650
K. pneumoniae (ATCC 10031)	–	700	750	350
P. vulgaris (ATCC 29905)	–	–	–	700
B. cereus (ATCC11778)	–	–	–	250
E. faecalis (ATCC 29212)	–	600	500	–
S. epidermidis (ATCC 12228)	–	625	350	200

reference antibiotics Amoxicillin and Cephalexin were observed.

The MIC values for the butanol, aqueous methanol, and essential oil extracts from *S. microcarpum* against all tested bacterial strains were determined and the results are shown in Table 4. Values ranged from 600–700 µg/mL for the butanol fraction, 350–750 µg/ml for the aqueous methanol fraction, and 200–700 µg/mL for the essential oil fraction. These data indicate that essential oil possesses striking inhibitory effects compared to butanol and aqueous methanol extracts. The MIC values obtained in this study support findings of the agar diffusion method. The MIC values of the aqueous methanolic and essential oil extracts exhibited the best inhibitory results against *Staphylococcus epidermidis* ATCC 12228, *Bacillus cereus* ATCC11778 and *Klebsiella pneumoniae* ATCC 10031.

CONCLUSIONS

The present study provides an evidence supporting the different chemical composition of the essnetial oil from different *Sedum* species, due to most likely climatic and genetic constraints. *S. microcarpum* extracts possess a broad spectrum of activity against a panel of bacterial strains responsible for the most common diseases. Further research is needed to determine the identity of the antibacterial compounds from this species and also to determine their spectrum of efficacy. However, the present study of *in vitro* antimicrobial evaluation of *Sedum microcarpum* extracts forms a primary platform for further phytochemical and pharmacological studies.

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Pharmacognostic and phytochemical evaluation of *Celosia argentea*

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ABSTRACT

Introduction: *Celosia argentea* Linn. (Amaranthaceae) is a common weed found abundantly in Bhor and Nagar districts of Maharashtra. The plant is traditionally used as an antidote for snake-poison, anti-ulcer and in the treatment of eye diseases, glandular swellings, eczema and constipation. The thorough microscopical details of the plant are not documented in any of the official literature hence the present study was undertaken with the view to establish the monograph of the plant. **Materials and Methods:** Macroscopy, Microscopy including transverse section, paradermal studies, powder characteristics, physicochemical standards were studied as per the protocol. The plant was also subjected to phytochemical Screening. **Results:** Microscopy of the leaves showed the thick, cylindrical and collateral vascular strand. The mesophyll tissue consists of adaxial band of vertically oblong cylindrical palisade cells, fan shaped and sandy balls crystals of calcium oxalate and anomocytic stomata. The stem shows unique vascular system sometimes called "anomalous type". Roots showed presence of primary concentric and secondary radial vascular bundle with distinct medullary rays running through the vascular bundles. The physicochemical parameters were estimated to define the purity limits. The preliminary phytochemical screening indicated the presence of carbohydrates, glycosides, flavonoids, tannins and steroids in the aerial parts, seed and root extracts which were confirmed by chromatography. **Conclusion:** Thus from the above study, it can be concluded that the pharamacognostic and phytochemical data could serve as a base for building the official monograph of the drug required for identification, isolation, synthesis of a new drug development in a ethanopharmacological research.

Keywords: *Celosia argentea,* chromatography, microscopy, morphology, pharmacognostic, phytochemical.

INTRODUCTION

Folk medicines mainly based on plants enjoy a respectable position today in the developing countries, where modern health service is limited. Safe, effective and inexpensive indigenous remedies are gaining popularity among the people of both urban and rural areas, especially in India and China. Information from indigenous traditional medicine has played a vital role in the discovery of novel products from plants as chemotherapeutic agents. Therefore the R&D thrust in the pharmaceutical sector is now focused on development of plant based drugs through investigation of leads.[1]

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Herbal medicines have two special characteristics which distinguish them from chemical drugs; use of crude herbs and prolonged usage. A single herb may contain a great many natural constituents and a combination of herbs even more. Experience has shown that there are real benefits in the long-term use of whole medicinal plants and their extracts, since the constituents in them work in conjunction with each other. However, there is very little research on whole plants because the drug approval process does not accommodate undifferentiated mixtures of natural chemicals, the collective function of which is uncertain. Moreover confirmation of the identity of the plant is a concern as many identical species having different set of chemical constituents may exhibit different pharmacological actions.[2]

Hence, WHO has set its guidelines for the assessment of herbal medicines (WHO/TRM/91.4) with respect to their identification, long-term use as well as their medical-historical and ethnological background through established monographs with the purpose to provide

scientific information on the safety, efficacy and quality control of widely used medicinal plants and to provide models for developing the monographs of unexplored species.[3]

Celosia argentea (Cockscomb) is an admired Asian, tropical, brilliant colored weed, documented for its vivid applications. It has diverse traditional uses ranging from anti-inflammatory, antimicrobial, immune modulating, hepatoprotective etc.[4,5] *Celosia argentea* is a herbaceous annual plant whose upright stem varies between 0.5 m and 1.5 m in height. Flowers are bisexual, pink or white in terminal elongated spikes of 2.5–15 by 2–2.5 cm, crowded, sometimes branched at the apex in a cock's comb form, ovate bracteoles, oblanceolate and curved.[6] This plant is raised from seeds and is widely grown in many regions of tropical Africa especially Nigeria, Benin and Congo Kinshasha. It is highly consumed as leafy vegetable because of the high nutritive value.[7,8]

The plant is enriched with natural products such as celogenamide A, celogentin A–D, H, J and K, moroidin, celosian, citrusin C, cristatain, nicotinic acid, betalains, amaranthin, isoamaranthin, betalamic acid etc. Though the plant has been thoroughly exploited for its phytochemical and pharmacological potential its pharmacognostic account remains unexplored. Thus the present research was undertaken to study the morphological, microscopical, physicochemical and phytochemical characters thoroughly with the purpose of contributing to the establishment of monograph for *Celosia argentea*.[6]

MATERIALS AND METHODS

Collection and drying

Fresh whole plants of *Celosia argentea* Linn. were collected from Bhor, district-Pune, Maharashtra and dried in the shade at room temperature.

Dried plant material was coarsely powdered in grinder and powder material was passed through 102 mesh, the coarse powder was used for extraction.

Authentication

The herbarium of the plant specimen was deposited at Botanical Survey of India, Pune; SVDAC1 (Reference number-BSI/WC/Tech/2011). The plant was authenticated by Majumdar S.C., Scientist, Joint Director, Botanical Survey of India, Koregaon Park, Pune.

Microscopy

The transverse sections of leaf, stem, seed and leaf paradermal (surface preparation) and microscopical evaluation were carried out at National Institute of Herbal Science, Chennai. The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of all the sections was 10–12 μm. De-waxing of the section was done by customary procedure. The sections were stained with toluidine blue as per the method of O'Brien. Since toluidine blue is a polychromatic stain, the staining results were remarkably good and rendered pink color to the cellulose walls, blue to the lignified cells, violet to the mucilage, blue to the protein bodies etc.

For studying the stomatal morphology, venation pattern and trichome distribution paradermal sections (sections taken parallel to surface of leaf) were taken and were cleaned with 5% Sodium Hydroxide or by peeling of epidermis by partial maceration employing Jeffrey's maceration fluid and then mounted using glycerin.[9,10,11]

Transverse sections of roots of plant were cleared using NaOH and mounted in glycerin after staining as per the method mentioned by Khandelwal, 2005 and stained with Phloroglucinol: Hydrochloric acid (1:1) and observed under microscope at 10X, 45X.[12]

The Photomicrographs of different magnifications were taken with Nikon Lbphoto-2 Microscopic unit. For normal observations the bright light was used. For the study of crystals, starch grains and lignified cells polarized light were employed. Since these structures have briefringent property they appear bright against the dark background. Descriptive terms of anatomical features are given in standard Anatomy books.[13]

Proximate analysis

Physicochemical constants such as moisture content, ash value, extractive value and foreign organic matter were studied using standard methods to determine its purity and stability.[14]

Determination of foreign organic matter

5 gm of air dried coarsely powdered drug was spread in a thin layer. The sample was inspected with the unaided eye or with the use of 5X lens. The foreign organic

matter was separated manually as completely as possible. Sample was weighed and percentage of foreign organic matter was determined from the weight of the drug taken.[14]

Determination of loss on drying

2 gm of sample was transferred to accurately weighed glass-stopper, shallow weighing bottle and sample was distributed evenly and poured to a depth not exceeding 10 mm. Then loaded bottle was kept in an oven for one hour. The sample was dried to constant weight. After drying it was stored at room temperature in a glass desiccators. The loss on drying was calculated in terms of percent w/w.[14]

Determination of ash value

Total ash

Accurately weighed 2 g of air dried crude drug was taken in a tared silica dish and incinerated at a temperature not exceeding 450°C until free from carbon, cooled and weighed. The percentage of ash was calculated with reference to the air-dried drug.[14]

Water soluble ash

The ash was obtained as per method described above and boiled for 5 minutes with 25 ml of water, filtered and the insoluble matter was collected on an ash less filter paper. It was further washed with hot water and ignited for 15 minutes at a temperature not exceeding 450°C and weighed. The difference in weight of incinerated and total ash represents the water-soluble ash. The percentage of water soluble ash was calculated with reference to the air-dried drug.[14]

Determination of acid insoluble ash

The ash was obtained as per method described above and boiled for 5 minutes with 25 ml of 2 M hydrochloric acid, filtered and insoluble matter was collected on an ash less filter paper. It was further washed with hot water and ignited for 15 minutes at a temperature not exceeding 450°C and weighed. The percentage of acid insoluble ash was calculated with reference to the air-dried drug.[14]

Determination of sulphated ash

The ash was obtained as per method described above. The residue moistened with 1ml Sulphuric acid. Gently heated until the white fumes were no longer evolved and ignited at 800 ± 25°C until all black particles disappeared. The crucible was allowed to cool and a few drops of Sulphuric acid were added and ignited cooled and weighed. Steps were repeated until two successive weighing do not differ by more than 0.5 mg.[14]

Determination of extractive value

Water soluble extractive value

Five gm of the air dried coarsely powdered drug was macerated with 100 ml of chloroform-water in a closed flask for 24 hours, and shaken frequently during first 6 hours then allowed to stand for 18 hours. It was filtered; 25 ml of filtrate was evaporated in a flat shallow dish, and dried at 105°C and weighed. Percentage of water-soluble extractive value was calculated with reference to air-dried drug.[14]

Alcohol soluble extractive value

Five gm of the air dried coarsely powdered drug was macerated with 100ml of ethanol in a closed flask for 24 hours, and shaken frequently during first 6 hours and allowed to stand for 18 hours. Then it was filtered, during filtration precaution was taken against loss of ethanol; 25 ml of filtrate was evaporated in a flat shallow dish, and dried at 105°and weighed. Percentage of Ethanol soluble extractive value was calculated with reference to air-dried drug.[14]

Similarly extractive values were also calculated by using the solvents- petroleum ether and chloroform.

Qualitative phytochemical screening

Powders of aerial parts, seeds and roots were extracted separately using continuous hot extraction (Soxhlet) method using 99% methanol as the solvent. These extracts were concentrated and subjected to phytochemical screening for the presence of flavonoids, sugars, tannins, glycosides and steroids.[15]

Thin layer chromatography of different extracts of *Celosia argentea*

The methanolic extract was subjected to TLC. The resolution of the TLC's was tried in different solvent systems and the best resolving solvent system was chosen for developing the plates. The plates were then exposed to Anisaldehyde- H_2SO_4/I_2 chamber/5% $FeCl_3$ in 0.1N HCl.[16,17]

Table 1. Organoleptic and macroscopical characters of *Celosia argentea*.

Serial No	Parameters	Observation		
		Leaf	Stem	Seed
1	Colour	Green with pink tinge	Light green with pink tinge	Polished, black and shining
2	Taste	Bitter	Bitter	Bitter
3	Shape	Linear-lanceolate, ovate, entire	Erect, simple, Branches grooved	Compressed and Oval
4	Surface	Smooth	Slightly lined	Smooth
5	Size	2.5–10 by 0.6–3.2 cm	0.3–0.9 m	1.0–1.8 mm

RESULTS AND DISCUSSION

Microscopy of leaf

Leaf

The leaf exhibits somewhat sinuous cross sectional outline [Figure 1]. The lamina is dorsiventral and midrib is prominent on the adaxial side and bulged into wide and thick, slightly lobed abaxial part. The midrib is 350 µm thick and 450 µm wide. The epidermal layer of the midrib is prominent, the cells being squarish and fairly thick walled. The ground tissue is three or four layered; the cells are thin walled and compact [Figure 2].

The vascular strand is a thick mass of two groups of xylem elements and prominent masses of phloem located on the abaxial part of the xylem. The xylem elements are wide, annular and thick walled. The lateral vein consists of flat adaxial side and thick appendage like outgrowth on the abaxial side [Figure 4]. The vascular strand is thick, cylindrical and collateral.

Lamina

The lamina is dorsiventral and amphistomatic (having stomata on both upper and lower side). The lamina is upto 160 µm thick. The abaxial epidermis is slightly thicker and cells are rectangular with thin cuticle [Figure 6 (A)]. The adaxial epidermis is fairly thick with cylindrical cells [Figure 6 (B)].

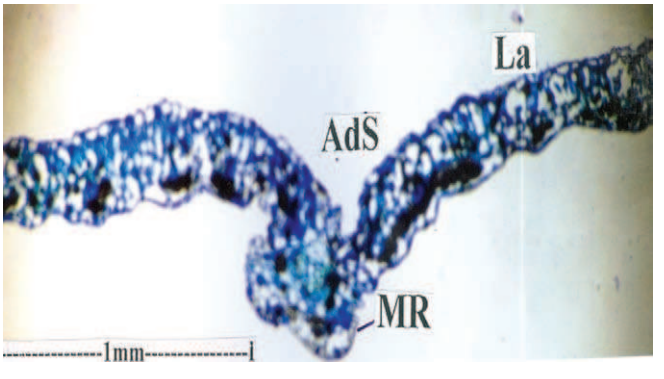


Figure 1. T.S. of leaf through midrib (10X).

The mesophyll tissue consists of adaxial band of vertically oblong cylindrical palisade cells and abaxial zone of about seven layers of lobed, loosely arranged spongy parenchyma.

The leaf margin is abruptly extended into thin conical end [Figure 4, 5]. The conical part is 50 µm thick; there are three layers of dilated cells.

Calcium oxalate crystals

Calcium oxalate crystals are abundant in the ground parenchyma. The crystals are sand like minute particles clumped

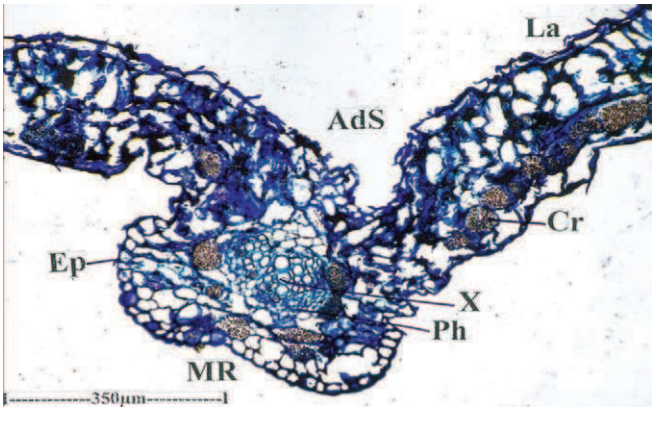


Figure 2. T.S. of leaf (40X) (Bright Field light).

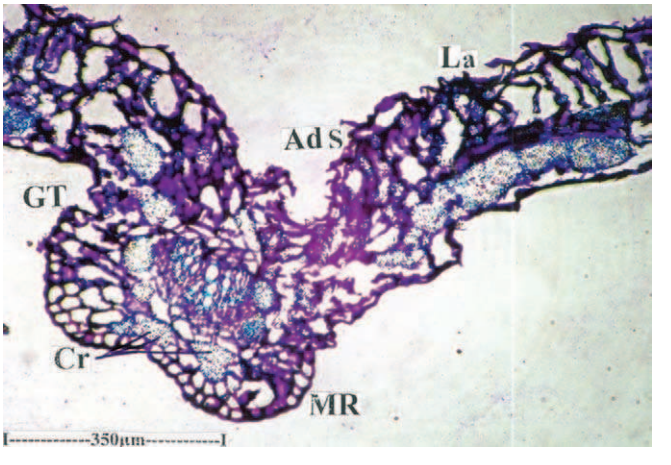


Figure 3. T.S. of midrib showing Calcium oxalate crystals (40X) (Polarized light).

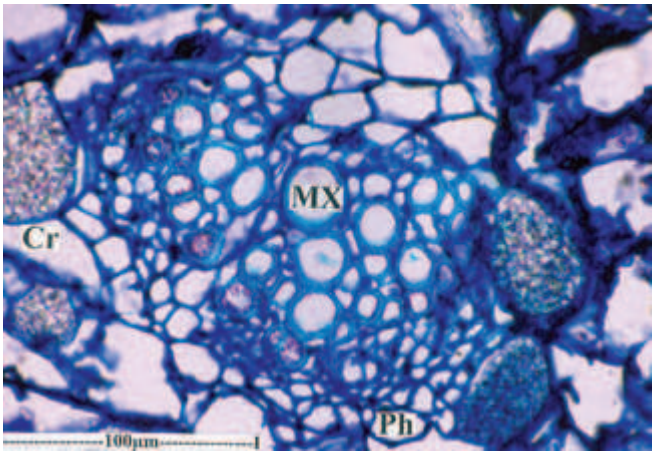


Figure 4. Vascular bundles of the midrib and sand crystals.

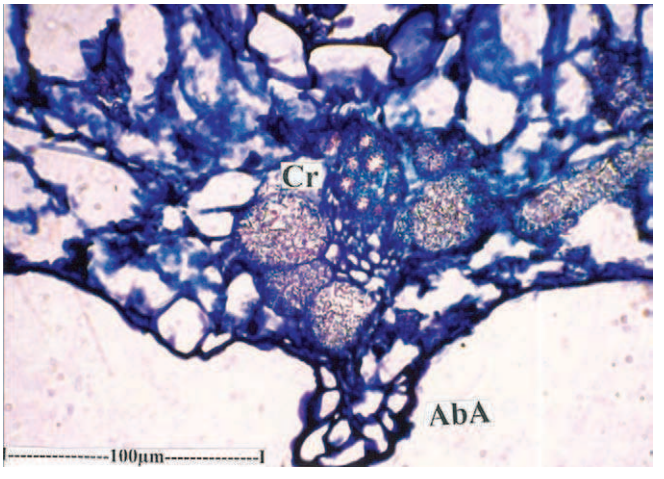
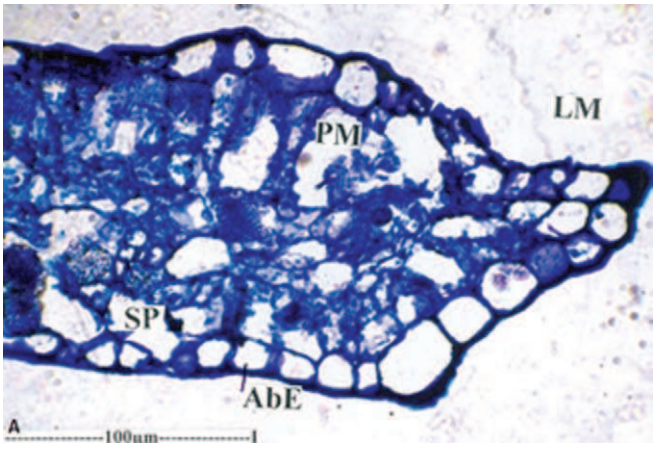


Figure 5. T.S. of lateral vein with Abaxial appendage.



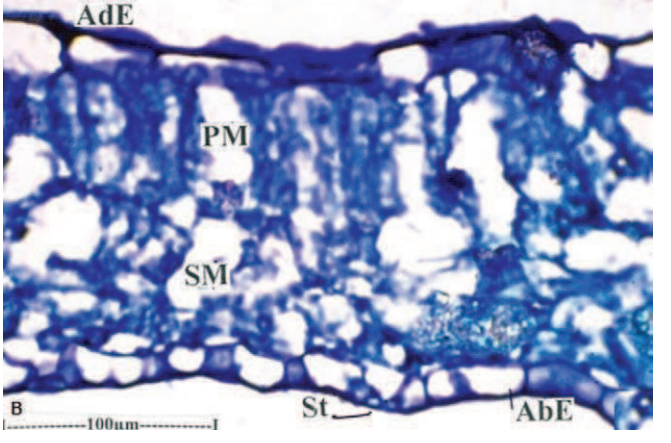


Figure 6. A], B] T.S. of Lamina (40X).

together into thick masses. The crystals fill the cells entirely [Figure 7 (B)]. The crystal bearing cells are slightly dilated; the cells are random in distribution. Some of the crystals are flat and lobed into wings [Figure 7 (A)].

Paradermal microscopy of the leaf

Epidermal cells and stomata

The epidermal cells of the lamina and the stomatal type were studied from the paradermal sections. The epidermal cells appear in surface view. They are polygonal in outline with straight, thick, anticlinal walls. The adaxial epidermal cells are slightly larger than the abaxial cells [Figure 8, 9].

The stomata are anomocytic type. There are no specific subsidiary cells around the stomata. The guard cells of the stomata on the abaxial epidermis are larger in size, measuring 40 × 40 µm. The guard cells on the adaxial epidermis are circular and are 25 × 30 µm.

In lamina the sand crystals of calcium oxalate are seen in lines of irregular orientation [Figure 10 (A)]. They occur in closed lines of dilated ground parenchyma cells [Figure 10 (B)].

Microscopy of stem

The stem is 1.7 mm in diameter. The cortex is developed into several irregular ridges all along its circumference. The ridges have parenchymatous cells. The stem consists of thick layer of epidermals which are rectangular in shape and have thick walls. The ground tissue is parenchymatous; the cells are fairly thick walled, wide, angular and compact. About three layers of cells inner to the epidermis are collenchymatous [Figure 12, 13). The vascular system is unique which is sometimes called "anomalous type". There is a thin cylinder of sclerenchyma at the outer zone of its pith. The sclerenchyma possesses small collateral vascular strands. Scattered in the pith are many prominent collateral strands. The outer vascular strands possess xylem fibres and vessels. The inner bundles which are called as medullary bundles are circular and contain xylem fibres

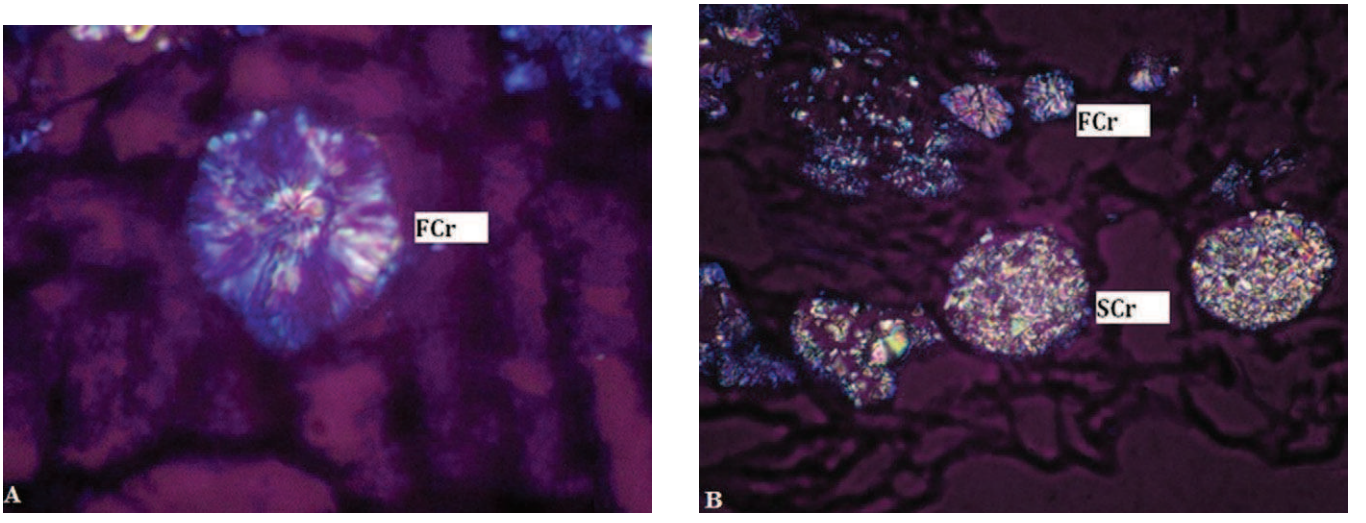


Figure 7. A] Enlarged fan-shaped crystal B] Presence of fan-shaped crystals of sand crystals in the mesophyll tissue (under polarized light).

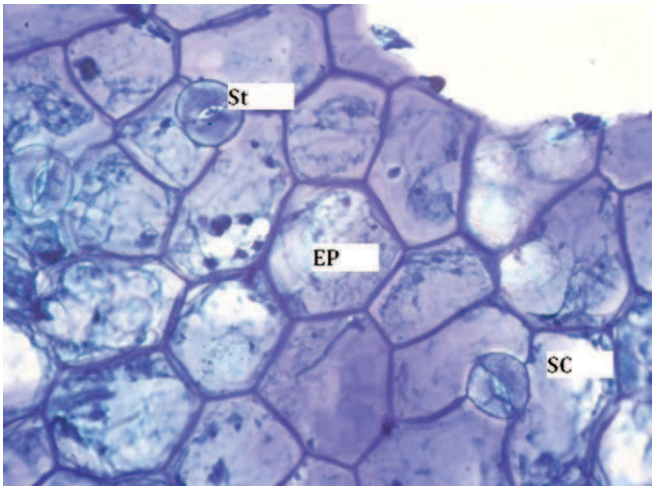
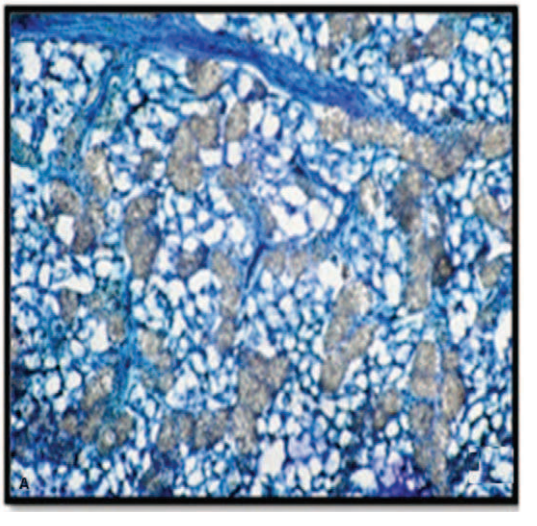


Figure: 8. Paradermal section of abaxial epidermis.



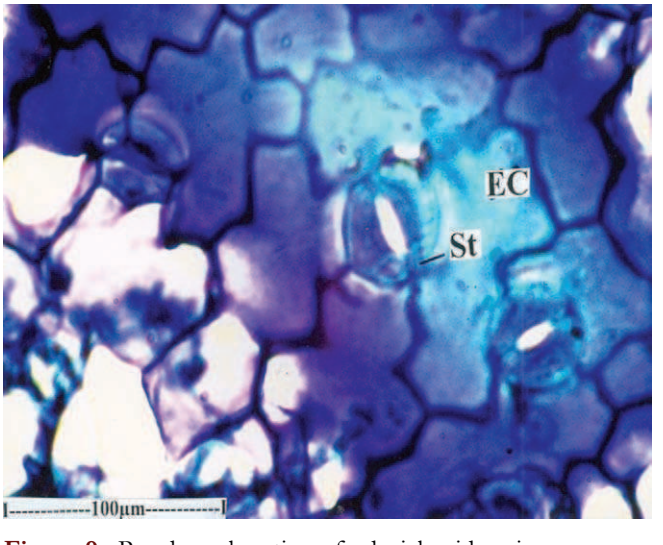


Figure 9. Paradermal section of adaxial epidermis.

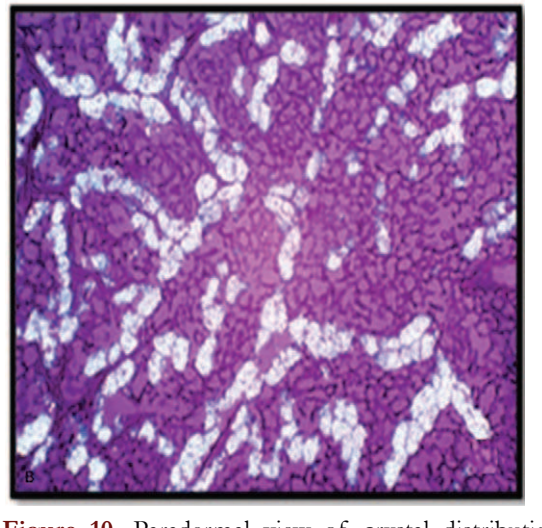


Figure 10. Paradermal view of crystal distribution pattern A: Bright field; B: Polarized light.

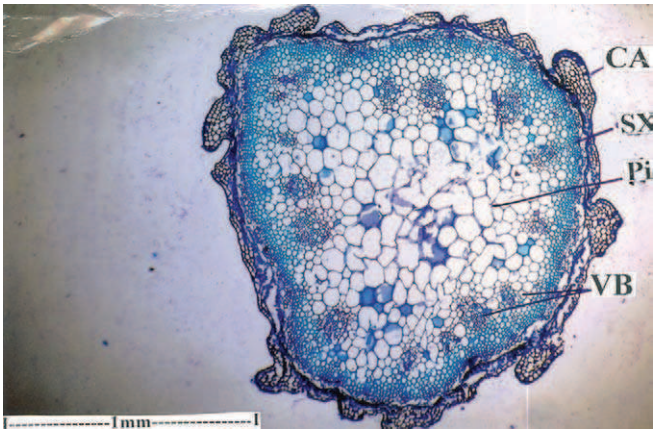


Figure 11. T.S. of Stem- Entire view (10X).

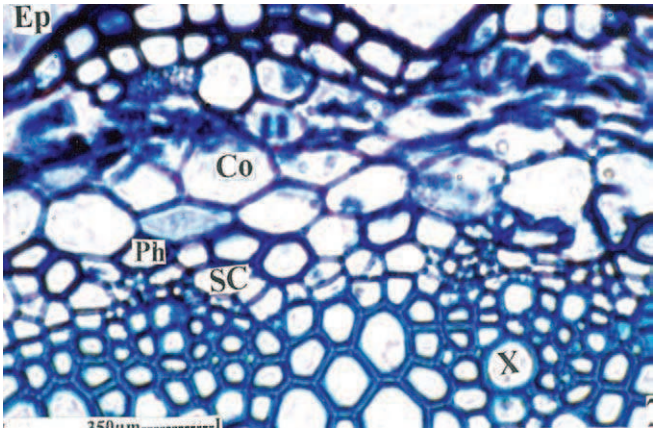


Figure 12. Cortex and outer cylinder of vascular tissue (40X).

Table 2. Physicochemical constants values.

Serial No.	Parameters	Aerial parts	Seeds	Roots
1.	**Determination of Foreign organic matter (% w/w)**	0.1	0.6	0.2
2.	**Determination of Moisture content(LOD) (% w/w)**	0.25	0.05	0.28
3.	**Ash values**			
	i) Total ash	14.0	10.0	11.3
	ii) Water-soluble ash	3.0	2.2	1.6
	iii) Acid-insoluble ash	2.0	1.8	1.0
	iv) Sulphated ash	20.2	15.3	12.6
4.	**Extractive values**			
	i) Water soluble extractive value(% w/w)	20.8	0.6	12.2
	ii) Alcohol soluble extractive value (%w/w)	7.1	1.2	5.6
	iii) Ether soluble extractive value (%w/w)	0.40	4.7	0.5
	iv) Chloroform soluble extractive value (%w/w)	3.8	4.1	3.2
	v) Methanol soluble extractive value (% w/w)	15.3	11.6	14.0

Table 3. Phytochemical Screening of Extracts.

Sr. No.	Test	Observation	Aerial part extract	Seed extract	Root extract
A]	**Test for Carbohydrates:**				
	i) Molish's Test	Violet ring	+	+	+
	ii) Fehling's Test	Brick red ppt.	+	+	+
	iii) Benedict's Test	Red solution	+	+	+
	iv) Barfoed's Test	Red ppt.	+	+	+
	v) Selwinoff's Test	Red solution	+	+	+
B]	**Test for Steroids:**				
	i) Salkowski Test	**$CHCl_3$ layer**- red **Acid layer**- greenish fluorescence	+	+	–
	ii) Libermann-Burchard Test	Red→blue→green	+	+	–
C]	**Test for Tannins:**				
	i) 5% $FeCl_3$ solution	Deep blue-black color	+	+	+
	ii) Lead acetate solution	White ppt.	+	+	+
	iii) Bromine water	Decoloration	+	+	–
D]	**Test for Flavonoids:**				
	i) Shinoda Test	Pink colour	+	+	+
	ii) With Lead acetate	Yellow ppt.	+	+	+
	iii) With 10% NaOH	Yellow solution→decolorises	+	+	+
E]	**Test for Proteins:**				
	i) Biuret Test	Violet/ pink color	–	–	–
	ii) Million's Test	Brick red ppt./ solution	–	–	–

Table 4. Thin Layer Chromatography of extract.

Sr. No.	Phytoconstituent	Solvent System	Detecting Agent	R_f value		
				Aerial	Seed	Root
1.	**Carbohydrates**	Benzene: GAA: Methanol (20:20:60)	Anisaldehyde- H_2SO_4	0.89	0.95	0.75
2.	**Glycosides**	Methanol: Water: Chloroform (65:25:4)	I_2 chamber	0.91 0.65	0.88	0.85
3.	**Flavonoids**	Ethyl acetate: Formic acid: GAA: Water (100:11: 11: 26)	Anisaldehyde	0.88 0.48	0.90	0.11
4.	**Tannins**	Ethyl acetate: Benzene (1:1)	5% $FeCl_3$ in 0.1N HCl	0.95 0.86	0.91	0.79
5.	**Steroids**	Toluene: Ethyl acetate (93:7)	I_2 chamber	0.68 0.90	0.92 0.67 0.48	0.65

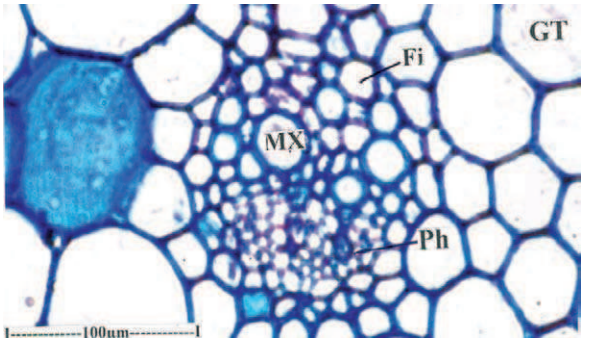


Figure 13. Central vascular strand (40X).

and prominent mass of phloem [Figure 13]. The vascular bundles are surrounded by parenchymatous pith cells.

Microscopy of root

Roots showed presence of primary concentric and secondary radial vascular bundle with distinct medullary rays running through the vascular bundles.

CONCLUSION

The present research work renders an elaborate account of various microscopical tissues present in different parts of *Celosia argentea* enabling it to be distinguished from other similar species. The physicochemical and phytochemical account of the plant will also help evaluate its quality and purity. Thus the work contributes in establishing the monograph of the plant.

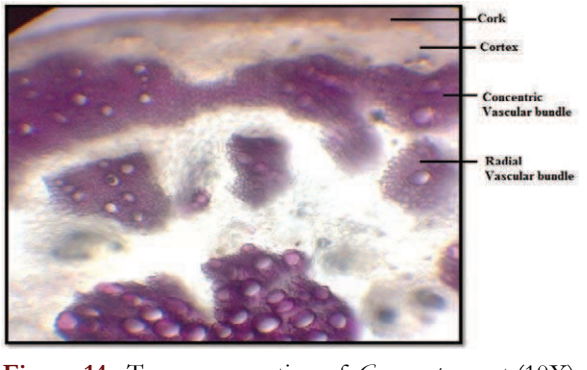


Figure 14. Transverse section of *C. argentea* root (10X).

CONFLICTS OF INTEREST

The authors report no conflict of interests.

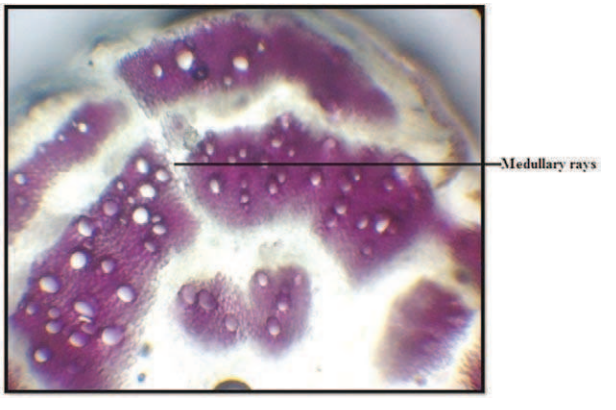


Figure 15. Transverse section of *C. argentea* root (40X).

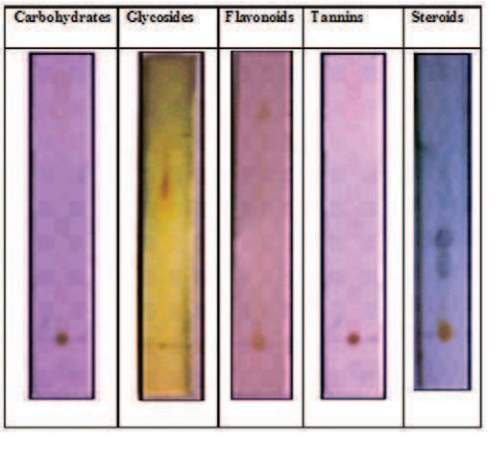


Figure 16. TLC of aerial parts extract.

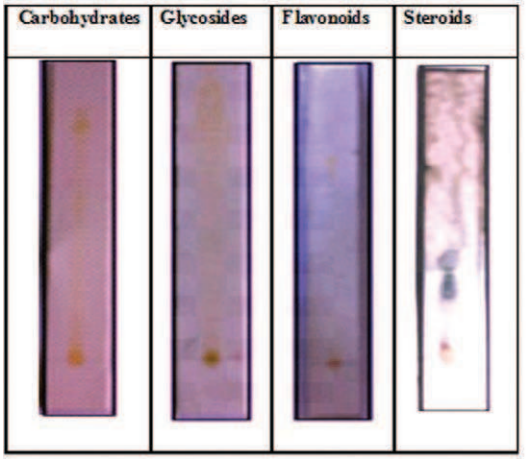


Figure 17. TLC of seed extract.

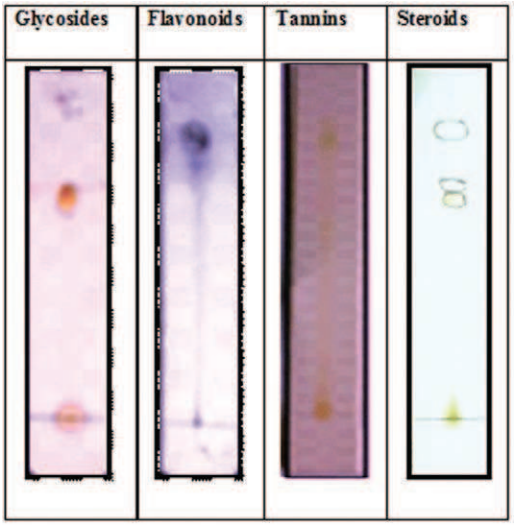


Figure 18. TLC of root extract.

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Pharmacognostic and Phytochemical studies on the rhizome of *Nardostachys jatamansi* DC. using different extracts

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ABSTRACT

Introduction: Nardostachys jatamansi DC. is an important ayurvedic plant. The root and rhizome contain active compounds with carminative, sedative, antispasmodic and tranquilizing properties. It is also used as an insect repellent. Essential oil from the drug shows antimicrobial activity against a number of microorganisms. **Method:** The present study comprises microscopic and macroscopic studies, phyto-chemical, physic-chemical, fluorescence, HPTLC analysis and microbial load estimation of the rhizome. **Conclusion:** Different extracts showed difference in presence of phyto-chemical constituents and physicochemical properties but chromatographic studies showed that all extracts contained valtrate. The study will provide referential information for the correct identification of the crude drug and establish pharmacopeial standards.

Keywords: *Nardostachy jatamansi* DC., *Valeriana jatamansi*, Pharmacognostic study, Phytochemical analysis, HPTLC Analysis, microbial load.

INTRODUCTION

Nardostachy jatamansi DC. belongs to Valerianaceae family and is also known as *Valeriana jatamansi*. It is distributed in Alpine Himalayas at an altitude of 3000–5000 m.[1] It is supposed to possess stimulant and antispasmodic properties. It is used in the treatment of epilepsy, hysteria, convulsive ailments, palpitations of the heart, diseases of the eye, itch, boils, swellings, diseases of the head, hiccough, etc.[2] Other use includes ailments of the hair. The roots are also used for improving the complexion, increasing the luster of the eye and promoting the growth and increasing the blackness of the hair.[3] Jatamansi consists of dried rhizome of *Nardostachy jatamansi* DC. It has been used in herbal combinations with other herbs to evaluate depressant activity.[4]

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According to ayurveda, the roots and rhizome of *Nardostachy jatamansi* DC. have various effects on 'doshas'. The plant is Vatashamak by snigdha; pittashamak by sheeta, tikta, kashaya, madhur, kaphashamak by tikta and tikshna. Ultimately it is tridoshashamak but especially kapha-pittanashak been clinically employed for their anti-ischemic, antioxidant, anticonvulsant, and neuroprotective activities. *Nardostachy jatamansi* DC. also works as a memory enhancer. Furthermore, it also reversed aging-induced amnesia due to natural aging of mice. Rhizome of Nardostachy *jatamansi* DC. is proved to be a useful memory restorative agent in the treatment of dementia seen in elderly persons.[5] The plant has been used as ethno medicine, perfume as incense and in modern medicine.[6]

The roots of the plant contain essential oil, rich in sesquiterpenes and coumarins. Jatamansone or valeranone is the principal sesquiterpene. Other sesquiterpenes include nardostachone, dihydrojatamansin, jatamansinol, jatamansic acid, jatamansinone, jatamansinol, oroseolol, oroselone, seselin, valeranal, nardostachyin, nardosinone, spirojatamol, jatamol A and B, calarenol, seychellene,

seychelane, coumarin: jatamansin or xanthogalin. A new sesquiterpene acid, nardin and new pyranocoumarin: 2, 2-dimethyl-3-methoxy-3, 4-dihydropyranocoumarin have been reported. Actinidine, an alkaloid has been reported.[7]

According to the World Health Organization, the macroscopic and microscopic description of a medicinal plant is the first step towards establishing the identity and the degree of purity of such materials and should be carried out before any tests are undertaken.[8]

The present study is an attempt to establish macroscopic and microscopic characteristics of a *Nardostachy jatamansi* DC. So as to standardize the rhizome and also to accomplish an analytical characterization of four extracts of *Nardostachys jatamansi* DC. by pharmacognostic and phytochemical analysis. The monograph of the plant thus prepared, is according to the WHO guidelines. These guidelines enable one to identify, authenticate, detect adulterants and standardize the plant material.[9]

MATERIAL AND METHOD

Dried rhizome was procured from local market in Mumbai. It was identified and authenticated at of SVKM'S Mithibai College, Vile Parle (WEST) Mumbai. Rhizomes were powdered using a mixer grinder and packed in air tight container. Dried rhizome was used for microscopic and macroscopic studies and rhizome powder was used for Phyto-chemical, Physicochemical, Fluorescence and HPTLC Analysis. The Microbial load was also determined using standard method.

Pharmacognostical evaluations were carried out taking free hand sections. The section of rhizome were cleared with chloral hydrate solution & then stained with Phloroglucinol and HCl and mounted in glycerin. These sections of the rhizome specimens were examined microscopically. Photomicrographs of these sections were recorded with MOTIC photomicroscope provided with MOTIC IMAGE PLUS 2.0 software.

Phytochemical analysis

The powdered rhizome was subjected to phytochemical screening for qualitative detection of phytoconstituents. From dried and coarsely powdered rhizomes extracts were prepared using methanol and hexane as solvent separately. Hot extraction was carried out in a soxhlet apparatus taking rhizome powder and solvent in ratio 1:6. The powder was extracted for 10–12 hrs. The cold extract of powdered rhizome was prepared by taking the solvent and powder in a conical flask and subjected to continuous agitation for 12 hrs on a shaker set at 250 rpm at the room temperature (25°C±2). Extracts were Filtered and evaporated to dryness and the residues were weighed. The percentage yields were calculated in terms of initial air dried plant material used for extraction. The colours of these extracts were noted. The extracts as mentioned above, were subjected to various qualitative phytochemical tests as per standard procedure,[10–11] for the identification of chemical constituents in the plant material.

Physicochemical analysis

Physicochemical properties such as the percentage of total ash, acid insoluble ash, water soluble ash, alcohol soluble and water soluble extractive values were determined as per the standard procedure.[12] Percentage of ash value is indicative of the purity of the drug and extractive values represent the presence of polar and non polar compounds.

Fluorescence analysis

Fluorescence study is an essential parameter for first line standardization of crude drug. The crude powder was subjected to these studies and the Fluorescence patterns were noted. The powdered materials were treated separately with different reagents and exposed to visible, ultraviolet light to study their Fluorescence behavior.[13] The colours obtained by application of different reagents at different wavelengths of radiation were recorded.

Determination of microorganisms

Medicinal plant materials carry a great number of bacteria and moulds, often originating from soil. A wide range of bacteria fungi form the naturally occurring microflora on herbs, aerobic spore forming bacteria also frequently predominate.[14] Current practices of harvesting, handling and production often cause additional contamination and growth of microflora. During the present study, exercise of determination of total viable count and detection of pathogens was performed as per WHO guideline on 'Quality Control methods for medical plant materials'.[15]

HPTLC analysis

Chromatographic finger-printing of phytoconstituents can be used for the assessment of quality, consistency and stability of herbal extracts or products by comparison of the standardized fingerprint pattern. A fingerprint has a potential to determine authenticity and reliability of herbal drugs and formulations.[16–17]

For HPTLC analysis samples were prepared by reconstituting 10 mg dry extract in 10 ml of solvent used for extraction. Chromatographic separation of hot and cold, methanolic and hexane extracts of *Nardostachy jatamansi* DC. rhizome were performed on 10 cm × 10 cm aluminum – backed HPTLC plates precoated with 200 µm layers of silica gel 60GF254 (Merck, Darmstadt. Germany). Hot and cold, methanolic and hexane extracts (10 µL each) were applied on to HPTLC plate as 8 mm wide bands and 12 mm apart from middle of bands by spray – on technique along with the nitrogen gas supply for simultaneous drying of bands. A CAMAG, Automatic TLC sampler 4 (ATS4) was used for this application. A constant spot application rate of 10 µL/sec was used to create band. After the application of bands, the chromatogram was developed, at room temperature (28 ± 2°C), in CAMAG glass twin-trough chamber 20 × 10 cm, previously saturated for 20 minutes with solvents pet ether: ethyl methyl ketone (8:2) (v/v) as a mobile phase.[18] The air-dried plates were directly viewed without derivatization in ultraviolet radiation. The chromatograms were scanned by densitometer at 254 nm with CAMAG TLC scanner and the Rf value was calculated with CAMAG win CATS planar chromatography manager software (version 1.4.2). The TLC plate was later derivatized with anisaldehyde sulphuric acid and heated at 105°C till bands developed. The developed plated were photo-documented at 254 nm and 366 nm.

RESULT

Macroscopic characteristics of rhizome

Dried rhizome is elongated, cylindrical in shape, 2.5–7.5 cm long and 1.5 cm thick, covered by bundles of fine brown fibers forming a network. These fibers are skeletons of sheathing leaf base, (Fig. 1). Outer layer of the rhizome is dark grey or brown in color whereas inner layer is slight brown or yellow, odour is highly agreeable, aromatic, taste is acrid, slightly bitter and aromatic. Rhizomes break easily and internally they are reddish brown in color.

Figure 1. Rhizome of *Nardostachys jatamansi* DC.

Microscopic character (Figs. 2–4)

Transverse section of rhizome is more or less circular in outline. Cork consists of outer most layers of irregular polygonal cells, filled with oil globules. Cortex is characterized by the presence of oleoresin cells. Phloem is in the form of patches of small cells. Cambium is distinct and continuous; xylem consists of vessels with pitted and scalariform thickenings. Tracheids are linear with scalariform thickenings, fibers are few in number. Medullary rays bi to multiserrate, not much prominent. Parenchymatous pith is present in the center. Starch grains abundantly present in groups.

Analysis of powdered drug (Figs. 5–7)

The powdered drug is light brown in colour, odour camphoraceous and the taste is bitter. It was cleared in chloral

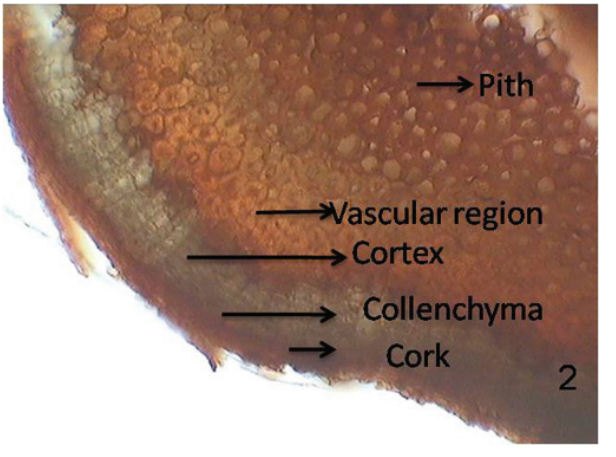

Figure 2. T.S of rhizome showing different regions of rhizome.

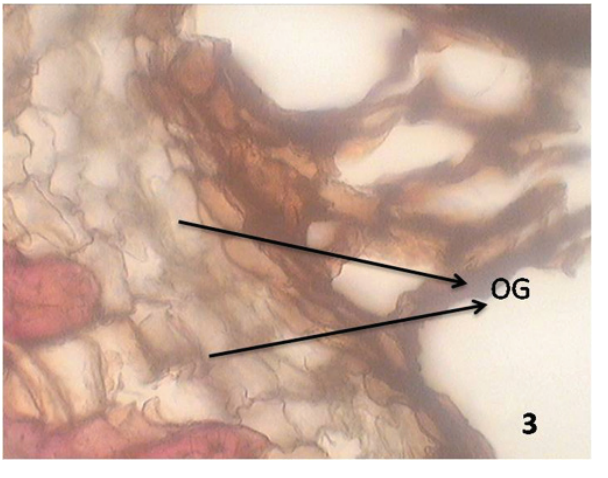

Figure 3. T.S of Rhizome showing oil globules in parenchymatous cell.

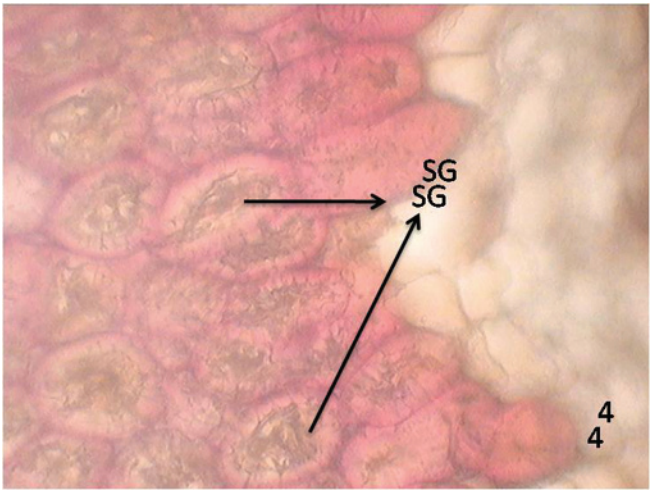

Figure 4. T.S of Rhizome showing starch grains in parenchymatous cell.

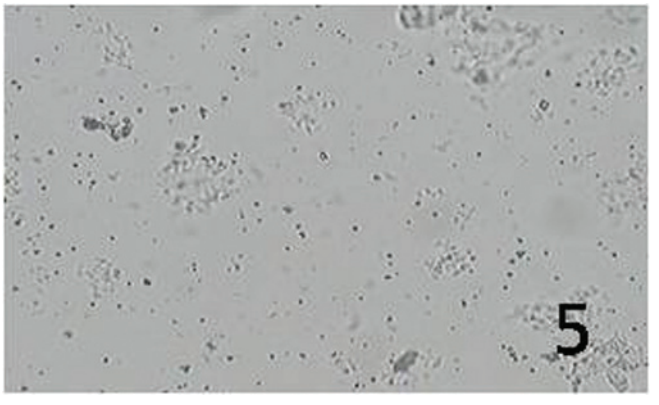

Figure 5. Starch grains.

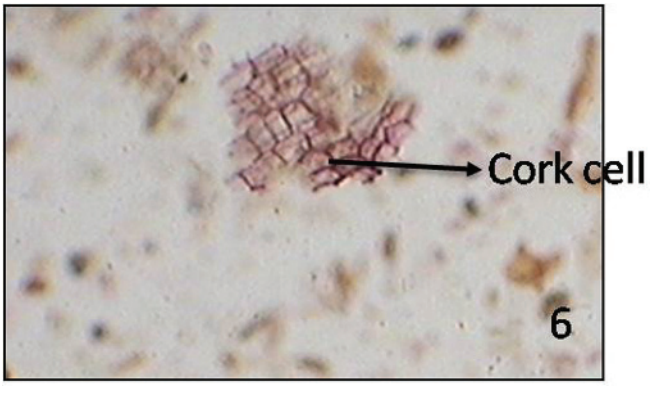

Figure 6. Cork cell.

hydrate solution and then stained with Phloroglucinol and HCl before mounting in glycerin. Under microscope the powder shows hairs, Medullary rays, Cells filled with reddish brown contents, fibers, Cork cells in surface view, Xylem vessels with pitted and scalariform secondary wall thickenings and starch grains.

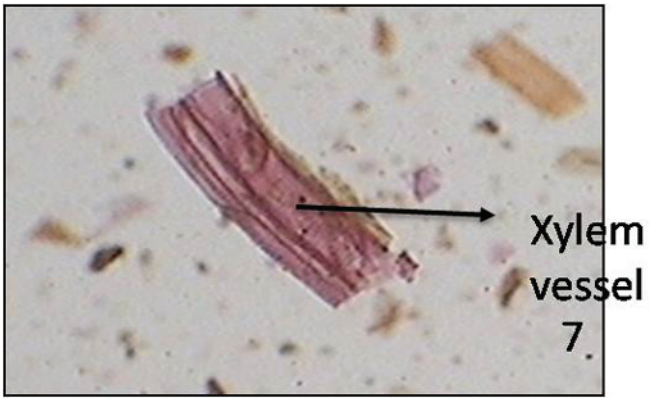

Figure 7. Xylem vessels.

Phyto-chemical analysis

Hot and cold methanolic extracts and hexane extracts were tested for different constituents; it reveals the presence of sugars, amino acids and tannins in both hot and cold methanolic extracts. Steroids were present in both hot and cold hexane extracts while alkaloids were found to be present in all four extracts.

Physico-chemical studies

The colour, consistency and percentage of extractive values for all four extracts were calculated. Hot methanolic extract (60–80°C) (dark brown, sticky mass, 5.63%), cold methanolic extract (brown, sticky mass, 1.33%), hot Hexane extract (40–60°C) (dark brown, sticky mass, 1.51%), cold hexane extract (greenish brown, sticky mass, 0.25%). Other physic-chemical characters are shown in (Table 1)

Determination of microorganisms

As already reported, *Nardostachy jatamansi* DC. has antimicrobial property.[19] Hence to neutralize the microbial growth or inhibitory substance present in the sample, a medium containing lecithin (0.1%) and Tween 80 (0.7%) was used for assessing microbial load of samples.

The test is employed to determine mesophilic bacteria and fungi, which grow under aerobic conditions. This is known to be as Total Aerobic Plate Count

Table 1. Results of Physico-chemical properties of dried rhizome powder of *Nardostachy jatamansi* DC.

Physico-chemical properties	Result (% w/w)
Total ash	20.53%
Acid insoluble ash	0.76%
Water soluble ash	1.005%
Alcohol soluble extractive	2.88%
Water soluble extractive	17.1%

(TAPC). The total aerobic plate count of *Nardostachy jatamansi* DC. rhizome powder was calculated under normal room temperature and in incubator at 37°C. No fungal propogules were observed in total fungal count (Table 2). The rhizome was also found to be free from objectionable pathogens such as *E .coli*, *Klebshiella, S.aureus*, etc.

Fluorescence analysis of extract and drug powder

The fluorescence analysis of the powdered drug of *Nardostachy jatamansi* DC. in various solvents and chemical reagents was performed under normal and UV light. The fluorescence was observed under UV long (365 nm) (Table 3).

Chromatographic Studies (Figs. 8–9)

HPTLC fingerprinting of extracts were accomplished with identical conditions to match the standard valtrate by comparing band of valtrate with Rf at 0.51.[18] HPTLC

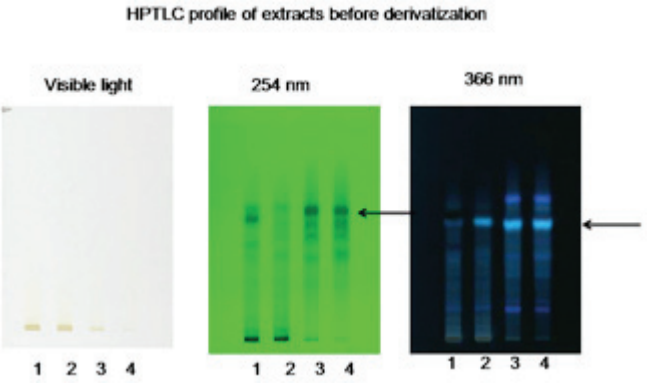

Figure 8. HPTLC profile of extracts of *Nardostachy jatamansi* DC. before derivatization.

fingerprinting revealed seven to eight phyto constituents separated at different Rf. Hot methanolic extracts gave 7 bands while Cold Hexane extract revealed 8 phyto-constituents. Both hot and cold Hexane extract showed 7 fractions. (Table 4) The band with Rf values 0.53 was observed in hot methanolic extracts and that of Rf 0.54

Table 2. Total viable count of dried rhizome powder of *Nardostachy jatamansi* DC.

Microbial method	Medium for plating	Microbial Counts	
		Room temperature (28± 2°C)	37°C
Total Aerobic Plate count	Nutrient Agar	6.4×10^5 cfu/ml	Too numerous to count
Total Fungal count	Sabourauds Dextrose Agar	No fungal propagules were observed	No fungal propagules were observed

Table 3. Fluorescence analysis of rhizome drug powder of *Nardostachy jatamansi* DC.

Treatment	Day light (UV)	Short wave (254 nm)	Long wave (365 nm)
Powder as such	Dark Brown	Black	No Fluorescence
Powder + Acetone	Chocolate Brown	Light Green	Faint Green Fluorescence
Powder + Conc. HCl	Brown	Dark Green	No Fluorescence
Powder + Conc. H_2SO_4	Chocolate Brown	Dark Brown	No Fluorescence
Powder + Conc. HNO_3	Chocolate Brown	Pale Green	No Fluorescence
Powder + Chloroform	Dark Brown	Dark Brown	No Fluorescence
Powder + Methanol	Brown	Pale Green	Green Fluorescence
Powder + 10% NaOH	Brown	Dark Green	No Fluorescence
Powder + 50% NaOH	Brown	No Colour	No Fluorescence
Powder + 5% KOH	Transparent Brown	Dark Green	No Fluorescence
Powder + 5% $FeCl_3$	Greenish Brown	Green	Yellow Orange
Powder + Ammonia	Dark Brown	Dark Green	No Fluorescence
Powder + pet. ether	Colourless	Colourless	No Fluorescence
Powder + acetic acid	Dark Brown	Light Green	Dark Brown
Powder + Dilute H_2O	Colourless	Slight Green	No Fluorescence
Powder + 95% Ethanol	Colourless	Colourless	Green Fluorescence
Powder + Dilute HNO_3	Brown Particles ,Colourless Liquid	Pale Green	No Fluorescence
Powder + Dilute HCl	Brown Particles ,Colourless Liquid	Pale Green	No Fluorescence
Powder + Dilute H_2SO_4	Brown Particles ,Colourless Liquid	Pale Green	No Fluorescence
Powder + Ethyl acetate	Brown	Colourless	No Fluorescence
Powder + Picric acid	Greenish yellow	Green	No Fluorescence
Powder + Methyl NaOH	Colourless	Faint Green	Green Fluorescence
Powder + Ethyl NaOH	Pale Brown	Faint Green	Green Fluorescence
Powder + 5% I_2	Reddish Brown	Dark Green	No Fluorescence

Table 4. Results for HPTLC of extracts of *Nardostachy jatamansi* DC.

Table i. HPTLC of hot methanolic extract

Sample	Solvent System developed	No. of peaks and Rf values
Methanolic extract	Pet ether: ethyl methyl ketone (8:2)	(7) 0.09 0.205 0.27 0.365 0.445 **0.53** 0.615

Table ii. HPTLC of cold methanolic extract

Sample	Solvent System developed	No. of peaks and Rf values
Methanolic extract	Pet ether: ethyl methyl ketone (8:2)	(8) 0.1 0.21 0.27 0.33 0.395 0.48 **0.54** 0.61

Table iii. HPTLC of hot hexane extract

Sample	Solvent System developed	No. of peaks and Rf values
Hexane extract	Pet ether: ethyl methyl ketone (8:2)	(7) 0.109 0.175 0.265 0.385 0.47 **0.54** 0.605

Table iv. HPTLC of cold hexane extract

Sample	Solvent System developed	No. of peaks and Rf values
Hexane extract	Pet ether: ethyl methyl ketone (8:2)	(7) 0.19 0.255 0.32 0.395 0.48 **0.54** 0.605

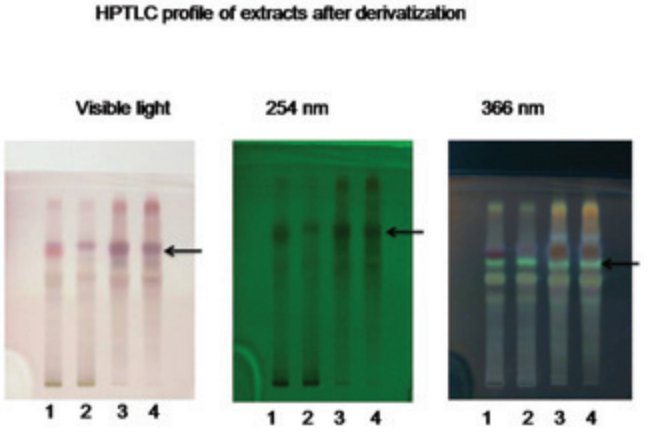

Figure 9. HPTLC profile of extracts of *Nardostachy jatamansi* DC. after derivatization.

in all three extracts, when scanned at 254 nm and 366 nm corresponds to valtrate standard.

DISCUSSION

Medicinal Plant material is obtained from different heterogeneous sources which may bring variation in therapeutic values and phyto-chemistry. Different parameters are used to assess the purity standards and authentication.[20] A morphological examination which includes macroscopic and microscopic characterization is preliminary study to decide authenticity of drug. Physico-chemical analysis, phyto-chemical analysis, fluorescence analysis and microbial load on the drug give qualitative information about the purity and the standard of the crude drug. The HPTLC fingerprinting profile is a very important parameter for herbal drug standardization and for proper authentication of the medicinal plants.

CONCLUSION

The data produced in the present investigation will also be helpful in the preparation of the drug's monograph for inclusion in various pharmacopoeias. The fingerprinting can also be a very important tool if any adulteration is suspected in the medicinal plant material. The present HPTLC-fingerprinting profile can be used as a diagnostic tool for the identity and to determine the quality and purity of the drug under investigation.

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Phytochemical screening and antimicrobial activity of ethanol and aqueous extracts of stem of *Glyphaea brevis* (spreng.) monachino on oral microorganisms

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ABSTRACT

The phytochemistry and antimicrobial activities of the aqueous and ethanolic extracts of the *Glyphaea brevis* stem was investigated. The results from this study indicate that the antimicrobial activities of the stem of *G. brevis* are microbe specific. The stem showed a significantly higher antimicrobial activity against *Staphylococcus aureus* and S*treptococcus mutans* even at a lower concentration of 3.13 mg/ml. Phytochemical screening of the plant part reveal the presence of alkaloids, flavonoids, anthraqiunones, saponins, steroids, cardiac glycosides, phlobotanins and carbohydrates in both extracts. While tannins and terpenoids were absent in extracts. The potency of the antimicrobial activities of *Glyphea brevis plant* has made the stem suitable for better dental care and cleansing.

Keywords: Potency, dental care, periodontal disease, medicinal plant.

INTRODUCTION

Studies have shown that chewing sticks are at least as effective as tooth brushes in maintaining oral hygiene.[1-4][5] Reported that Africans that use chewing sticks have fewer carious lessons than those using tooth brushes, and their use has been encouraged by the World Health Organization.[6] Most of these chewing sticks have been shown to have significant antimicrobial activity against a broad spectrum of microorganisms.[7] Described the activity of several plant extracts against *Streptococcus mutans*, a carcinogenic organism. Since then, several investigators including[8][9] as well as[10] have made similar reports of the antimicrobial activity of chewing stick extracts.

Secondary metabolites flavonoids, lignoids and tannins are well distributed in the plant kingdom. Their structures prevent oxidative damage, many of their beneficial effects in animal and human organisms are reported as anti-aging, anti-inflamation,[11] anti-carcinogenic[12–13] anti-mutagenic[14][15] anti-ulcer and anti-artherogenic effects and as the inhibitors of human low density lipoprotein oxidation.

Recent interests in chewing sticks and their extracts have focused on their effects on organisms that are involved in oral infections.[16] Have reported the isolation of compounds active against aerobic and anaerobic periodiotopathic bacteria from *Ceanothus americanus* plant employed by Native Americans in the treatment of these conditions.[17] Also reported some Nigerian chewing sticks exhibited strong activities against a broad spectrum of bacteria including those that are involved in both medical and dental morbidity. The study also showed that some of the chewing stick extracts demonstrated activity against antibiotic resistant organisms. So they can be viewed as sources of novel lead substances with potential therapeutic or preventive application.

Utilization of non-timber forest products (NTFP) is gaining importance in the tropical world because of their commercial importance to the host community.[18] They further stated that chewing sticks are important NTFP widely used for dental cleaning in tropical Africa. Chewing sticks also impact varying taste and sensation such as a tingling peppery taste, a bitter taste and numbness.[19][20] Posited that chewing sticks, in addition to providing

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mechanical stimulation of the gums, also destroy microbes; these advantages of the chewing sticks over the conventional toothpaste and brush has been attributed to the strong teeth of Africans.[21]

[18]Reported that some of the chewing sticks being used are obtained from the following plants: *Garcinia manni, Musalaria accuminita, Terminalia glaucescens, Anogeissus leiocarpus, Pseudocedrela kotschyi, Zanthoxyllum gilleti* and *Azadirachta indica*.

Investigation further revealed that some of these chewing sticks possess anti-microbial activity against oral microbial flora such as *Staphylococcus aureus* and *S. auricularis,*[18] *Candida albicans, Aspagillus flavus, Microsporium gypseum* and *Trichophyton metagrophytes.*[22]

Benefits derived from using medicine obtained from plants are that they are relatively safer than synthetic alternative by offering profound therapeutic benefits and more affordable treatment.[23–24]

It has been found that some drugs are synthesized from plants, and it is estimated that plant materials are present in, or provide the models for more than 50% of western drugs.[25] Chewing sticks with antimicrobial activity could become a potential source of new drugs for oral diseases. Oral microorganisms are known for their pathogenesis in tooth decay, gingivitis, periodontitis, and their ability to cause teeth loss.[26]

The twigs of *G. brevis* are chewed to clean teeth. *G. brevis* of the family tiliaceae are herbs, shrubs and trees, they are mostly found in tropical region. They are tap rooted with tall and erect stem may be simple or branched with mucilage. The flowers are regular, bisexual or hypogenous; the leaves are stipulate, alternate, simple, entire or toothed. It is valued as vegetable[27] and put into various therapeutic uses such as treatment of hepatitis and poisoning,[28] has anticonvulsant properties.[29] *G. brevis* possess antioxidant properties which are related to the therapeutic activities of the plant.[30] Plants constitute highly available and low-cost sources of antioxidants.

OBJECTIVES OF THE STUDY

This study is aimed at screening the phytochemistry and evaluates the antimicrobial activities, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) levels of the aqueous and alcoholic extracts of *G. brevis* used as chewing stick against oral microorganisms which are responsible for dental caries.

MATERIALS AND METHODS

Collection and identification of plant material

The stems of *Glyphea brevis* plants were collected from Edo North Senatorial District of Edo State. The plants were identified by Dr J. F Bamidele of the Department of Plant Biology and Biotechnology, University of Benin, Benin City, Edo State.

Preparation and extraction of plant material

The fresh stems of *Glyphaea brevis* were cut from the plants, rinsed in water and spread on trays and dried under the sun. The plant materials were then transferred to the oven set at 45°C for 20–30 minutes before being reduced to fine powder with the aid of a mechanical grinder. The powdered plant materials were then collected and stored in a tightly covered glass jars and kept for further studies.

For ethanol extraction, 100 g of the powdered stem and root materials were soaked in 600 mls of ethanol. The resultant solution was filtered using Whatman filter paper No 1 after 48 hours under room temperature (25°C). For aqueous extraction, 100 g of the powdered stems and root materials were boiled in 600 mls of water for 24 hours after which the resultant solutions were filtered using Whatman filter paper No 1.

The two extracts were concentrated through evaporation process using a water bath set at 100°C. The extracts were then stored in a refrigerator until required for use.

Preparation of stock solution of extracts

Fresh stock (known concentration) solution of the extract was prepared for each experiment. To prepare a required concentration of the extract, a specific weighed amount of the concentrated extract was dissolved completely in an appropriate volume of distilled water. To prepare 100 mg/ml concentration of extract, 1 gm of either of the extract was dissolved in 10 ml of distilled water in a sample bottle, corked and shaken vigorously to obtain a homogenous solution.

Phytochemical screening

The phytochemical tests were carried out on the aqueous and ethanol extracts using standard procedures as described by.[31][32]

Antimicrobial investigation

Source of microorganisms

Pure stock cultures of *Staphylococcus aureus, Staphylococcus auricularis, Streptococcus pyogenes, Streptococcus mutans, Candida albicans, Aspergilus flavus, Microsporium gypseum* and *Bacillus subtilis* isolated from patients with dental diseases were obtained from the Department of Medical Microbiology, Department of Dentistry University of Benin, and University of Benin Teaching Hospital (UBTH). These pure isolates were used and maintained in slants of Nutrient Agar (NA), Blood Agar (BA) and Potato Dextrose Agar (PDA) at 4°C until when needed for further studies.

Microbial inoculums preparation for susceptibility testing

The inocula of the bacterial isolates were prepared by growing each pure isolate in nutrient broth at 37°C for 24 hrs. The fungal isolates were grown in Potato dextrose broth at 28±2°C for 48 hrs.

After incubation, 1 ml of the diluted cultures of the microbial isolates in normal saline using a Pasteur pipette was inoculated unto the solidified nutrient agar at 40°C for bacteria and Potato dextrose agar for fungi.

Antimicrobial assay

Antimicrobial activity was evaluated by noting the zone of inhibition against the test organisms.[33] Two colonies of a 24-hour plate culture of each organism were transferred aseptically into 10 ml sterile normal saline in a test tube and mixed thoroughly for uniform distribution. A sterile cotton swab was then used to spread the resulting suspension uniformly on the surface of oven-dried Nutrient agar, blood agar for bacteria and Potato dextrose agar plates for fungi, respectively. Three (3) adequately spaced wells of diameter 4 mm per plate were made on the culture agar surface respectively using a sterile metal cup-borer. 0.2 ml of each extract and control were put in each hole under aseptic condition, kept at room temperature for 1 hour to allow the agents to diffuse into the agar medium and incubated accordingly. Conventional antibiotics were used as positive controls for bacteria and fungi respectively; distilled water was used as the negative control. The plates were then incubated at 37°C for 24 hours for the bacterial strains and at 28°C for 72 hours for fungal isolates. The zones of inhibition were measured and recorded after incubation. Zones of inhibition around the wells indicated antimicrobial activity of the extracts against the test organisms. The diameters of these zones were measured diagonally in millimeter with a ruler and the mean value for each organism from the triplicate cultured plates was recorded. Using the agar-well diffusion technique, an already made gram positive and gram negative (Asodisks Atlas Diagnostics, Enugu, Nigeria) standard antibiotic sensitivity disc bought from a laboratory chemical equipment store in Benin city was used as positive control for bacteria while Ketoconanzone was used as positive control for fungi. Distilled water was used as negative control for all the test organisms.

Determination of minimum inhibitory concentrations (MICs) of the extracts

The lowest concentration of the extracts that will inhibit the growth of test organisms is the Minimum Inhibitory Concentration (MIC). The initial concentration of the plant extract (100 mg/ml) was diluted using double fold serial dilution by transferring 5 ml of the sterile plant extract (stock solution) into 5 ml of sterile Normal saline to obtain 50 mg/ml concentration.[34] Different concentrations were prepared from the crude extract by doubling dilution in distilled water. The different concentrations were 50, 25, 12.5, 6.25, 3.125, 0.625, and 0.3125 mg/ml respectively. Each dilution was introduced into nutrient agar plates, blood agar plates and potato dextrose agar plates already seeded with the respective test organism. All test plates were incubated at 37°C for 24 hrs for bacteria and 28°C ± 2°C for 72 hrs for fungi. The minimum inhibitory concentration (MIC) of the extracts for each test organism was regarded as the agar plate with the lowest concentrations without growth.[33]

Minimum bactericidal concentration (MBC)

The Minimum Bactericidal Concentration (MBC) of the plant extracts were determined by the method described by.[35–36] Samples were taken from plates with no visible growth in the MIC assay and subcultured on freshly prepared nutrient agar plates, blood agar plates and Potato dextrose agar plates and later incubated at 37°C for 48 hours and 28 ± 2°C for 72 hours for bacteria and fungi respectively. The MBC was taken as the concentration of the extract that did not show any growth on a new set of agar plate.

Determination of the antibiotic susceptibility of bacteria isolates

The disc diffusion method[37] was used for the determination of microbial sensitivity. The antibiotic discs employed were: septrin, chloranphenicol, sparfloxcarcin, ciprofloxacin, amoxicillin, augmenting, gentamicin,

pefoxacin, ofloxacin, streptomycin, zinnacef and recophin. The zones of inhibition were measured and interpretation was in accordance with manufacturer's instructions.

RESULTS

In Table 1 the results of the phytochemical analysis of aqueous and ethanolic stem extracts of *G. brevis* revealed the presence of some secondary metabolites such as alkaloids, flavonoids, cardiac glycosides, anthraquinones, phlobotannins, steroids, Carbohydrates and Saponins. Tannins and terpenoids were absent in the aqueous and ethanol extract of *G. brevis*.

Table 2 shows the antimicrobial properties of the ethanol extract of the *G. brevis* on the test microorganisms. All the test organisms were sensitive to the ethanol extracts at a concentration of 100 mg/ml. The activities of the ethanol extracts on all the tested organisms were significantly different from one another. The highest zone of inhibition was recorded against *A. flavus* with a sensitivity diameter of 15.13 ± 0.09 mm, while the least sensitive was recorded against *M.gypseum* with a sensitivity diameter of 6.63 ± 0.03 mm.

Plant extracts were more susceptible to *A. flavus* (fungus) followed by *S. auricularis* (gram +ve), *S. mutans* (gram +ve), *B. subtilis* (gram +ve rod bacteria), *C. albicans* (fungus), *S. pyogenes* (gram +ve), *S. aureus* (gram +ve) and *M. gypseum* (fungus) respectively. Table 2 also revealed that the antimicrobial activity of the aqueous extract of *G. brevis* plant extracts were significantly different from one to another on each organism. *G. brevis* (stem) did show antimicrobial activity in all the tested oral microorganisms with the highest sensitivity of 13.80 ± 0.06 mm against *M. gypseum* and the least sensitivity of 4.20 ± 0.12 mm against *S. auricularis* at the test concentration of 100 mg/ml.

Table 2 indicates the comparison of the effect of the aqueous and ethanol extracts of *G. brevis* on the test organisms. It was revealed that the ethanol extract has the highest antibacterial and antifungal activity against all the tested oral microorganisms with inhibition diameters of 14.03 ± 0.03mm (*S. auricularis,* gram +ve bacteria) and 15.13 ± 0.09 mm (*A. flavus,* fungus) respectively at 100 mg/ml.

Table 3 revealed that only *S.mutans* was sensitive to the plant extract at the test concentration of 3.125 mg/ml with inhibition zone of 2.87 ± 0.12 mm, it also revealed the highest susceptibility when compared with other test organisms at all concentrations except at 12.5 mg/ml and 100 mg/ml where *S. aureus* showed the highest zone of inhibition of 5.73 ± 0.07 mm, while *A. flavus* recorded the highest susceptibility to the plant extract at the highest concentrations of 100 mg/ml with inhibition zone of 13.37 ± 0.09 mm closely followed by *S. auricularis* with inhibition zone of 13.20 ± 0.12 mm.

Table 4: present the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) values of the ethanol extracts of *G. brevis*. The ethanol extract of the plant at various concentrations showed minimum inhibitory concentration (MIC) at 6.25 mg/ml against *S. mutans* and *S. aureus*, in addition the extract indicate MIC at 12.50 mg/ml against *S. auricularis* and 25.00 mg/ml extracts against *S. pyogenes*, and *B. subtilis*, while for the fungi 25 mg/ml extracts of

Table 1. Phytochemical screening of the aqueous and ethanol extracts of *G. brevis* plant stem used as chewing stick.

	Test Plant Extracts									
	Chemical components									
G. brevis (stem)	Cardiac									
	Alkaloids	Flavonoids	Anthraquinones	Saponins	Tanins	Glycosides	Steroids	Terpenoids	Phlobatanins	Carbohydrates
Aq	+	+	+	+	–	+	+	–	+	+
Et	+	+	+	+	–	+	+	–	+	+

KEY: + = Present, – = Absent, Aq = Aqueous, Et = Ethanol

Table 2. Zone of inhibition of Aqueous and Ethanol extracts (100 mg/ml) of *G. brevis* against selected oral pathogens.

	Test Organisms							
G.brevis (stem)	*S. aureus*	*S. auricularis*	*M. gypseum*	*S. pyogenes*	*S. mutans*	*B. subtillis*	*A. flavus*	*C. albicans*
Aq	6.01 ± 0.03	4.20 ± 0.12	13.80 ± 0.06	5.20 ± 0.58	5.37 ± 0.72	6.67 ± 0.07	7.00 ± 0.06	5.00 ± 0.06
Et	9.13 ± 0.03	14.03 ± 0.03	6.63 ± 0.03	10.50 ± 0.06	12.27 ± 0.09	11.30 ± 0.09	15.13 ± 0.09	11.00 ± 0.58

NB: Means ± S.E.M; n = 3, Means ± S.E.M within a row are significantly different, $P < 0.01$.
– = No inhibition. Aq = Aqueous, Et = Ethanol

Table 3. Zone of inhibition in mm of various concentrations of the ethanol extract of *G. brevis* on test organisms.

Test organisms	Concentration of extract (mg/ml)						Sterile distilled water
	3.125	6.5	12.5	25	50	100	
S. aureus	–	3.27 ± 0.09	5.73 ± 0.07	7.30 ± 0.12	8.63 ± 0.09	10.03 ± 0.15	–
S. auricularis	–	–	3.30 ± 0.06	4.70 ± 0.12	6.37 ± 0.12	13.20 ± 0.12	–
S. pyogenes	–	4.03 ± 0.09	5.13 ± 0.09	6.53 ± 0.09	7.47 ± 0.09	10.30 ± 0.06	–
S. mutans	2.87 ± 0.12	4.13 ± 0.09	5.30 ± 0.12	7.57 ± 0.09	9.13 ± 0.09	11.23 ± 0.09	–
M. gypseum	–	–	–	3.23 ± 0.09	4.70 ± 0.06	5.87 ± 0.09	–
B. subtilis	–	–	–	3.03 ± 0.12	3.50 ± 0.06	5.23 ± 0.09	–
A. flavus	–	2.10 ± 0.06	3.10 ± 0.07	5.10 ± 0.09	8.53 ± 0.03	13.37 ± 0.09	–
C. albicans	–	–	–	3.10 ± 0.06	4.57 ± 0.09	5.47 ± 0.09	–

NB: Means ± S.E.M; n = 3; Means ± S.E.M within a row are significantly different, P < 0.01.
– = No inhibition.

Table 4. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) in mg/ml of the ethanol extracts of *G. brevis* stem against the test organisms.

	Test bacteria				
***G.brevis* (stem) (mg/ml)**	***S. aureus***	***S. auricularis***	***S. pyogenes***	***S. mutans***	***B. subtillis***
MIC	6.25	12.50	25.00	6.25	25.00
MBC	12.50	25.00	25.00	25.00	25.00

NB: Values are means + S.E.M (n = 3);
Values within a row with different alphabet are significantly different, P < 0.01

Table 5. Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) in mg/ml of the ethanol extracts of *G. brevis* plant against the test fungi.

	Test fungi		
***G. brevis* (stem) (mg/ml)**	***M. gypseum***	***A. flavus***	***C. albicans***
MIC	3.125^{a}	12.5^{b}	25.00^{c}
MFC	6.25^{a}	25^{b}	50.00^{c}

NB: Values are means + S.E.M (n = 3);
Values within a row with different alphabet are significantly different, P < 0.01;

G. brevis was against *C. albicans*. The MBC value was lowest with 6.25 mg/ml against *S. mutans*.

Table 5: Indicate the least MIC value of 3.125 mg/ml against *M. gypseum* a fungus and the MFC value of 6.25 mg/ml of the plant extract against *M. gypseum* and higher concentrations against other fungi.

Table 6: shows the activity of the commercial antibiotics (standard sensitivity disc) on the test bacteria. It revealed a sensitivity zone of inhibition diameter varying from 4.0 mm – 28.3 mm against the bacterial isolates used.

Table 7 revealed that ketoconanzone (commercial fungi antibiotic) was active against all the test fungi. It had the highest activity against *M.gypseum* with inhibition diameter of 26 mm, followed by *C. albicans 24 mm* and *A. flavus* 17 mm.

DISCUSSION

The result of qualitative phytochemical screening tests carried out on the aqueous and ethanol extracts of stem

Table 6. Sensitivity zone of inhibition of commercial antibiotics (standard sensitivity disc) on the test bacteria.

	Zone of inhibition (in mm) for commercial antibiotics									
Test isolates	**CN**	**APX**	**R**	**CPX**	**E**	**SXT**	**PEF**	**OFX**	**S**	**AM**
Staph. Aureus	28.3	7.4	–	24.5	9.7	–	21.6	27.0	–	–
Staph. Auricularicus	27.0	4.6	–	20.9	7.7	–	17.8	28.1	–	10.5
S. pyogenes	22.0	4.1	–	17.9	7.8	–	13.4	20.1	–	9.4
Strept. Mutans	20.6	4.0	–	18.7	8.8	–	14.5	20.9	–	–
B. subtilis	24.8	5.5	–	19.0	8.6	–	11.8	19.9	–	7.7

PEF = Pefloxacin (10 µg/ml), CN = Gentamicin (20 µg/ml), APX = Ampiclox (30 µg/ml)
OFX = Ofloxacin (10 µg/ml), AM = Amoxacillin (30 µg/ml), R = Rocephin (25 µg/ml)
CPX = Ciprofloxacin (10 µg/ml), S = Streptomycin (30 µg/ml), SXT = Septrin (30 µg/ml)
E = Erythromycin (10 µg/ml), APX = Ampiclox (10 µg/ml), – = No inhibition

Table 7. Sensitivity zone of inhibition of commercial fungi antibiotics (ketoconanzone) on the test fungi.

Test fungi	Ketoconanzone (200 mg/ml)
Aspergillus flavus	17 mm
Candida albicans	24 mm
Microsporium gypseum	26 mm

of *G. brevis* revealed the presence of alkaloids, flavonoids, steroids, anthraquinones, cardiac glycoside, saponins, phlobotannins and carbonhydrates while absent of tannins and terpenoids were observed in both extracts.

The presence of bioactive compounds has been known to show medicinal activity as well as exhibit and regulate some physiological activity.[38–39] Saponins have been reported to be an antifungal agent;[40] reported the importance of steroids as potent starting material in the synthesis of sex hormones. Some of these bioactive compounds could be said to be responsible for the antimicrobial activity observed in this study. The presence of phytochemicals in plants has been shown to be responsible for the therapeutic activity of plants.[31]

The aqueous and ethanol extracts of *G. brevis* used showed inhibitory activities against all the test organisms (Table 2 and 3). It was observed that susceptibility increased with increased concentration of the extracts (Table 3). Of all the tested organisms, *A. flavus* recorded the highest susceptibility of 15.13 ± 0.09 mm for ethanol extract while *M. gypseum* recorded the highest susceptibility of 13.80 ± 0.06 mm for aqueous extract both at concentration of 100 mg/ml.

The ethanol extracts exhibited more activity, potency and consistency than the aqueous extract. These results support earlier studies which observed that plant extracts in organic solvent provided more consistent antimicrobial activity compared with those extracted in water.[41–42]

The most sensitive test bacterium was *S. auricularis* in ethanol extract with zone of inhibition of 13.20 ± 0.12 mm at the highest concentration of 100 mg/ml, while the aqueous extract was most active against *M. gypseum* a fungus with zone of inhibition of 13.80 ± 0.06 mm at 100 mg/ml (Table 2 and 3).

Ethanol extract recorded the highest antifungal activity of 15.13 ± 0.09 against *A. flavus* at 100 mg/ml, while *C. albicans* was the least sensitive fungi among the test organisms (Table 2).

The inhibitory effects of the various concentration of ethanol extracts of the plant against *S. Aureus, S. Auricularis, M. gypseum, S. mutans, B. subtilis, A. flavus* and *C. albicans* (Table 3), were significantly even different at the same concentration of the extracts.

It was also observed that the extracts were active when compared with the negative control (sterile distilled water) against all the test organisms (Table 3). The control recorded no visible activity. The positive control (standard sensitivity disc) used on the test bacteria revealed that gentamycin, perfloxacin, ampiclox, ofloxacin, ciprofloxacin, and erythromycin had inhibitory effects on all the test bacteria (Table 6). Amoxacillin has activity against S. *auricularis, S. pyogenes* and *B. subtilis* with inhibitory diameter of 10.5, 9.4 and 7.7 mm respectively. *S. aureus* was more sensitive to the commercial antibiotics with zone of inhibition of 28.3 mm for gentamycin, 27.0 mm for ofloxacin, 24.5 mm for ciprofloxacin and 21.6 mm for perfloxacin.

Rocephin, streptomycin and septrin showed no inhibition zone on any of the tested organisms. Comparatively, the ethanol and aqueous extracts can be said to possess better activity than these since they contain both pharmacological and non-pharmacologically active substances as oppose to the pure active substances contained in the control antibiotics. The effect of the commercial antifungal drug (Ketoconanzone) tested at a concentration of 200 mg/ml against the test fungi (Table 7) can be considered not better in activity when compared with the extracts, particularly at the highest tested concentration of 100 mg/ml which was two times lower in concentration than that of the fungal antibiotics. This probably implies that if the concentrations of the extracts were increased, it could lead to increased activity. The MIC values of the ethanol extracts of the plant are given in Table 3.

Antimicrobial substances can be considered as bactericidal agents when the ratio MBC/MIC ≤ 4 and bacteriostatic agents when the ratio MBC/MIC > 4.[43] For the ethanol extracts tested, the ratio MBC/MIC was ≤ 4 against all the tested bacteria. It suggests that the extracts can be considered as possessing bactericidal effect (Table 4 and 5).

In a related development,[20] posited that chewing sticks, in addition to providing mechanical stimulation of the gums, also destroy microbes; these advantages of the chewing stick over conventional toothpaste and brushes has been attributed to the strong teeth of Africans.[21]

CONCLUSION

Dentists are scarce in many parts of Africa, particularly in rural areas. Although diet plays a major role in preventing

dental caries, the practice of dental hygiene is also important. While toothpaste and toothbrushes are widely used by a sector of the population with a high level of formal education, toothpaste consumption is still low and chewing sticks are still in common use in many parts of Africa, particularly in West Africa. Even when people would prefer to use toothbrushes, they do not have access to toothpaste due to high cost or remoteness. Therefore, continued access to popular and effective sources of chewing sticks with anti-bacterial and anti-fungal properties is important as a primary health care measure. The results from these studies provide evidence for the ethnomedicinal uses of the tested plant as chewing sticks. The presence of bioactive substances (secondary metabolites) in the plant could be responsible for their medicinal properties. Although the actual biochemicals could not be ascertained in the scope of the present study, the extracts (ethanol and aqueous) of the plant showed activity against all the test bacteria and fungi. This implies that the plant used for this study possesses both antibacterial and antifungal properties. Therefore, it is recommended as potential chewing sticks for the reduction of dental caries due to the presence of antimicrobial, antifungal and phytochemical agents.

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Inhibitory effect of *Anacyclus pyrethrum* extract on acetylcholinesterase enzyme by *invitro* methods

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ABSTRACT

Aim: The present study was conducted to evaluate acetylcholinesterase (AChE) inhibitory effects of *Anacyclus pyrethrum*. **Materials and Methods:** The hexane, chloroform and ethanolic extract of *Anacyclus pyrethrum* were tested for their *invitro* anticholinesterase inhibitory effect by spectrophotometeric and by TLC bioassay method. **Results:** The result revealed that ethanolic extract of *Anacyclus pyrethrum* showed better anticholinesterase inhibition compared to other extracts. The most active one was found to be ethanolic extract of *Anacyclus pyrethrum* having IC_{50} value at 70 ± 1.52 mg/ml. Hexane extract of *Anacyclus pyrethrum* has not shown any anticholinesterase inhibitory effect. Chloroform extract of *Anacyclus pyrethrum* was found to have IC_{50} value at 150 ± 3.68 mg/ml and rivagistmine was found to have IC_{50} value to be 350 ± 5.95 mg/ml. TLC bioassay is an easier and rapid means for detection of enzyme inhibition. The hexane, chloroform and ethanolic extract of *Anacyclus pyrethrum* were tested for their anticholinesterase inhibition by TLC bioassay. **Conclusion:** TLC bioassay is an easier and rapid means for detection of enzyme inhibition. The active spots appeared as white spots on yellow background. Ethanolic extract of *Anacyclus pyrethrum* showed more than one active spots compared to other extracts. The TLC assay also demonstrated AchE inhibitory activity for ethanolic extract of *Anacyclus pyrethrum*.

Keywords: *Anacyclus pyrethrum,* acetylcholinesterase, Alzheimer's disease, TLC bioautography.

INTRODUCTION

Alzheimer's disease (AD), is a complex, multifactoral, progressive, neurodegenerative disease primarily affecting the elder population and is estimated to account for 50–60% of dementia cases in persons over 65 years of age.[1,2] The impairment of central acetylcholine (ACh) neurotransmission due to neural degeneration is believed to be a principal neuropathological feature of Alzheimer's disease. Based on the cholinergic hypothesis that memory impairments in patients suffering from AD result from a defect in the cholinergic system, an important approach to treat this disease is to enhance the acetylcholine level in the brain by inhibition of the enzyme acetylcholinesterase (AChE).[3] The treatment with drugs which increase cholinergic neurotransmission causes an improvement in cognitive deficits in AD.[4] Since AD has become a public health burden, and the commonly available synthetic drugs have undesirable side effects, new treatment strategies based on medicinal plants have been the subject of current focus. *Anacyclus pyrethrum* (AP), family Asteraceae is used in traditional system of medicine and it is regarded as a tonic to the nervous system.[5] The roots contain anacyclin, pellitorine, hydrocarolin, inulin, traces of volatile oil and seasamin. *Anacyclus pyrethrum* is a perennial, procumbent herb, which is found throughout India. The plant roots are reported for antinflammatory,[6] immunostimulating,[7] and anabolic, aphrodisiac activities.[8] However its cognitive improvement potential remains to be explored. Therefore present study has been undertaken to

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investigate acetylcholinesterase (AChE) inhibitory effects of *Anacyclus pyrethrum*, For this intention, we have tested *in vitro* anticholinesterase action of the *Anacyclus pyrethrum* by spectrophotometeric and by TLC bioassay method.

Plant material

The roots of *Anacyclus pyrethrum* used for investigation was collected from hilly regions of Pathanamthitta district of Kerala and the roots of *Anacyclus pyrethrum* was identified and authenticated for their correct botanical identity by Professor P. Jayaraman, Director, National Institute of Herbal Science, Chennai (Ref. no: PARC/-2009/419) and samples (voucher no: 0997) of the plant has been be deposited in the herbarium of the institute.

Chemicals

5,5-Dithio-bis(2-nitrobenzoic) acid, Acetylthiocholine iodide, Acetylcholinesterase electric eel, were obtained from Sigma Aldrich. All other chemicals were of analytical grade obtained from SD fine chemicals Ltd.

Preparation of extracts

The powdered roots of *Anacycluspyrethrum* were subjected to successive soxhlet extraction with different solvents such as hexane, chloroform and ethanol in the increasing order of polarity. The obtained solvent extracts were evaporated under reduced pressure using rotary vaccum evaporator. Extracts were weighed and percentage was calculated in terms of the air-dried weight of the root material. The yield of the petroleum ether, chloroform and ethanol extract was found to be 8.53%, 5.66%, 7.81% w/w respectively.

Preliminary phytochemical screening

The extracts of *Anacyclus pyrethrum* root was subjected to preliminary phytochemical screening.[9]

Determination of anticholinesterase activity

AChE inhibitory activity of the extracts was measured by the spectrophotometric method.[10] Acetylcholinesterase was used, while acetylthiocholine iodide was employed as substrate of the reaction. 5,5-Dithio-bis(2-nitrobenzoic) acid (DTNB) was used for the measurement of the cholinesterase activity. Hydrolysis of acetylthiocholine iodide was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by the enzyme at a wavelength of 412 nm utilizing, UV–visible recording spectrophotometer, Shimadzu (Japan). Percentage of inhibition of AChE was determined by comparison of rates of reaction of samples relative to blank sample using the formula $(E-S)/E\times100$, where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were done in triplicate. Rivastigmine was used as reference.[11]

Estimation of IC_{50} values

The concentrations of test samples that inhibited hydrolysis of the substrate (acetylthiocholine) by 50% (IC_{50}) were determined by monitoring the inhibitory effect of extracts with increasing concentrations in the assays.

Thin layer chromatography (TLC) with bioassay detection for AChE inhibition

The TLC with bioassay detection for AChE inhibition was studied.[12] A 2.5 mm silica gel plate was used as stationary phase. The plant extracts were spotted in the TLC plate it is developed in the mobile phase toluene: ethylacetate (97:3). After the plate was developed it was dried at room temperature and then sprayed with 30 mM acetylthiocholine followed by 20 mM DTNB. The plate was dried at room temperature for 45 minutes and then sprayed with AChE. After 20 minutes the plate was observed under visible light. A positive test indicating AChE inhibiton was colorless spot on the yellow background.

RESULTS

Preliminary phytochemical tests

Preliminary phytochemical analysis of hexane, chloroform and ethanolic extract of *Anacyclus pyrethrum*. Phytochemical analysis of hexane revealed the presence of phytoconstituents such as carbohydrate, sterols, tannins, phenols, terpenes. Phytochemical analysis of chloroform extract revealed the presence of phytoconstituents such as alkaloids, tannins, terpenes. Phytochemical analysis of ethanolic extract revealed the presence of phytoconstituents such as alkaloids, carbohydrate, tannins, phenols, flavanoids, glycoside and saponins.

Invitro anticholinesterase inhibitory activity (Estimation of IC_{50} values)

The hexane, chloroform and ethanolic extract of *Anacyclus pyrethrum* were tested for their *invitro* anticholinesterase inhibitory effect at 62.5, 125. 250, 500, 1000 and 2000 µg/ml concentrations. Inhibitory activity on acetylcholinesterase for the hexane, chloroform and ethanolic extract of Anacyclus pyrethrum were evaluated and percentage inhibition was calculated. The most active one was found to be

ethanolic extract of *Anacyclus pyrethrum* having IC_{50} value at 70 ± 1.52 µg/ml.Hexane extract of *Anacyclus pyrethrum* has not shown any anticholinesterase inhibitory effect. Chloroform extract of *Anacyclus pyrethrum* was found to have IC_{50} value at 150 ± 3.68 µg/ml and rivagistmine IC_{50} value was found to be 350 ± 5.95 µg/ml. Results are shown in Tables 1, 2, 3, 4.

Table 1. *Invitro* anticholinesterase inhibitory activity of hexane extract.

Hexane extract (µg/ml)	%anticholinesterase inhibition	IC_{50} Value((µg/ml)
62.5	NI	–
125	NI	
250	NI	
500	2.41	
1000	7.25 ± 0. 02	
2000	9.5 ± 0. 32	

NI-Non inhibition

Table 2. *Invitro* anticholinesterase inhibitory activity of chloroform extract.

Chloroform extract (µg/ml)	%anticholinesterase inhibition	IC_{50} Value((µg/ml)
62.5	20 ± 0.01	150 ± 3.68
125	37 ± 0.02	
250	75 ± 0.01	
500	79 ± 0.12	
1000	83 ± 0.02	
2000	90 ± 0.32	

Table 3. *Invitro* anticholinesterase inhibitory activity of ethanolic extract.

Ethanolic extract (µg/ml)	%anticholinesterase inhibition	IC_{50} Value((µg/ml)
62.5	47 ± 0.01	70 ± 1.52
125	76 ± 0.02	
250	79 ± 0.01	
500	84 ± 0. 12	
1000	85 ± 0. 02	
2000	87 ± 0. 32	

Table 4. *Invitro* anticholinesterase inhibitory activity of rivastigmine.

Rivastigmine (µg/ml)	%anticholinesterase inhibition	IC_{50} Value((µg/ml)
62.5	15 ± 0.01	350 ± 5.95
125	32 ± 0.02	
250	39 ± 0.01	
500	73 ± 0. 12	
1000	75 ± 0. 02	
2000	80 ± 0. 32	

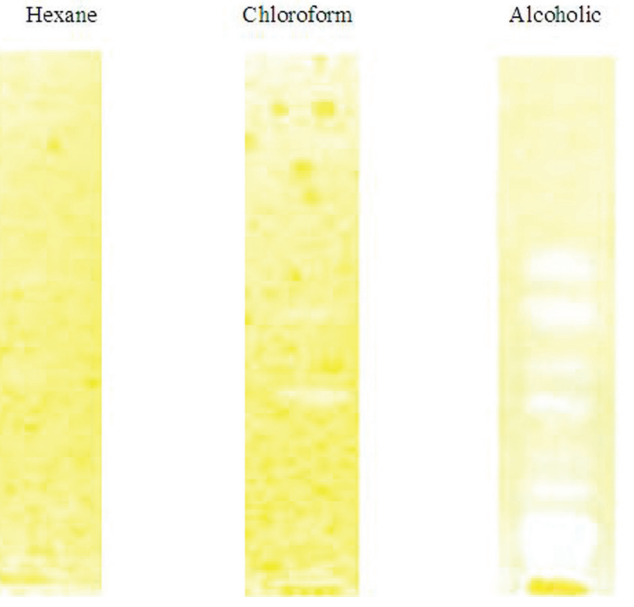

Figure 1. Thin layer chromatography (TLC) with bioassay detection for AChE inhibition.

Thin layer chromatography (TLC) with bioassay detection for AChE inhibition

TLC bioassay is an easier and rapid means for detection of enzyme inhibition. The hexane, chloroform and ethanolic extract of *Anacyclus pyrethrum* were tested for their anticholinesterase inhibition by TLC bioassay. TLC bioautography of active extract revealed active spots on TLC. Ethanolic extract of *Anacyclus pyrethrum* showed better anticholinesterase inhibition compared to other extracts. The active spots appeared as white spots on yellow background. Ethanolic extract of *Anacyclus pyrethrum* showed more than one active spots compared to other extracts. The TLC assay demonstrated AchE inhibitory activity for ethanolic extract of *Anacyclus pyrethrum*. Results are shown in Fig. 1.

DISCUSSION AND CONCLUSION

Preliminary phytochemical analysis of hexane, chloroform and ethanolic extract of *Anacyclus pyrethrum* revealed the presence of phytoconstituents such as carbohydrate, sterols, tannins, phenols, alkaloids, terpenes, carbohydrate, flavanoids, glycoside and saponins. Alzheimer disease is the most common form of neurodegenerative disorders, neurochemically characterized by a consistent deficit in cholinergic neurotransmission. For this reason, symptoms can be treated by the use of agents that restore the level of acetylcholine through inhibition of cholinesterase, AChE. In late stages of AD, levels of AChE decline by up to 85%,

in the brain. Recently, the inhibition of this enzyme was targeted as a new approach to intercede in the progression of AD.[13] The most important strategy to increase cholinergic function is inhibition of acetylcholinesterase. AChE inhibitor is always the target of many Alzheimer dementia drugs.[14] Therefore *Anacyclus pyrethrum* was evaluated by activity by TLC bioautography and by *invitro* methods. *Invitro* anticholinesterase inhibitory study showed that chloroform and ethanolic extract of *Anacyclus pyrethrum* exhibited dose dependent *invitro* anticholinesterase inhibitory effect. From the study, among the different extracts tested for *Anacyclus pyrethrum* it was found that the ethanolic extract of *Anacyclus pyrethrum* indicated higher anti-AChE activity than chloroform extract. Ethanolic extract of *Anacyclus pyrethrum* showed IC_{50} value at 70 ± 1.52 µg/ml. TLC bioautography of extracts also revealed that ethanolic extract of *Anacyclus pyrethrum* showed significant anticholinesterase inhibition compared to other extracts. TLC bioautography of active extract revealed active spots on TLC. The active spots appeared as white spots on yellow background. Ethanolic extract of *Anacyclus pyrethrum* showed more than one active spots compared to other extracts. The TLC assay demonstrated AchE inhibitory activity for ethanolic extract of *Anacyclus pyrethrum*. In vitro analysis confirmed cholinesterase-inhibiting properties for the ethanolic extract of *Anacyclus pyrethrum*. The *in vitro* results, indicates that any effect of *Anacyclus pyrethrum* on improving memory could be due to cholinestrerase inhibitory activity and improving the levels of acetylcholine. The phytoconstiuents present in the ethanolic extract has to be isolated to prepare a ideal drug for Alzheimer's disease.

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In vitro activity of some medicinal plants from Cachar district, Assam (India) against *Candida albicans*

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ABSTRACT

Introduction: The current research has focused on the potentiality of medicinal plants for treatment of *Candida albicans* infections. Five plants viz. *Clerodendron colebrookianum* Walp. (Leaf), *Gnetum gnemon* L. (Leaf), *Sarcochlamys pulcherrima* (Roxb.) Gaud. (Leaf), *Garcinia lancifolia* (Don) Roxb (Leaf) and *Euryale ferox* Salisb. (Seed), used as traditional medicines in Cachar district, Assam, India were selected to evaluate *in vitro* activity against *C. albicans*. **Methods:** The plant samples were extracted with methanol. Agar well diffusion assay was used to test the activity of the plant extracts and broth microdilution method was used to determine the MIC. **Results:** All extracts showed anticandidial activity with zones of inhibition ranging from 17 to 25 mm at 2×10^5 µg/ml extract. *E. ferox* and *S. pulcherrima* showed the highest activity with the MIC value of 1.25×10^4 µg/ml. The remaining extracts were comparatively less effective showing MIC value of 2.5×10^4 µg/ml. **Conclusions:** Anticandidial activity of the plants extracts, observed in this study highlighted further *in vivo* investigation and identification of the active compounds for therapeutic uses. The anticandidial activity of *S. pulcherrima* and *G. lancifolia* is probably the first report to the best of our knowledge.

Keywords: Anticandidial activity, *Euryale ferox*, *Garcinia lancifolia*, *Sarcochlamys pulcherrima.*

INTRODUCTION

Candida species produce a wide spectrum of diseases, ranging from superficial mucocutaneous disease to systemic candidiasis. Among the Candida species, the most predominant species is *Candida albicans*.[1] Clinico-mycological profile from the Northeast India also revealed that *C. albicans* was the common pathogen amongst the *Candida* species.[2] Emergence of multi drug resistant strains and failure of existing antifungal agents to cure *Candida* infections especially *C. albicans* is now a therapeutic challenge. Within the limited data available, an increased incidence of invasive candidiasis, aspergillosis and zygomycosis was reported by Chakrabarti et. al. (2008) of which the invasive candidiasis is the most common opportunistic mycosis.[3]

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Cachar district of Assam, India, an excellent reservoir of flora, together with the traditional information on their medicinal uses acts as a natural resource to target antimicrobial agent. Medicinal plants used traditionally could be one potent source for such antifungal agents for treatment of *Candida* infections, as evidenced from the results of antimicrobial screening of medicinal plants, reported from time to time. Selection of plants, based on ethnopharmacological perspective enhances the probability of success in new drug discovery efforts.[11,12] Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for the development of new drugs.[13] Results of the extensive research on herbs, carried out throughout the world, unfurled the potentiality of developing antimicrobial agents from the plants.[4–6,8–10]

In the present study we have considered *Clerodendron colebrookianum* Walp. (Leaf), (Verbenaceae), *Gnetum gnemon* L. (Leaf), (Gnetaceae) *Sarcochlamys pulcherrima* (Roxb.) Gaud. (Leaf), (Urticaceae) *Garcinia lancifolia* (Don) Roxb (Leaf), (Clusiaceae)and *Euryale ferox* Salisb. (Seed),

(Nymphaeaceae) against *Candida albicans*. The plants were chosen on the basis of traditional information on medicinal uses by the tribal people of Cachar district of Assam. Moreover, the selection criteria included edibility of the plant parts and thus our research methodology will rise above the possibility of toxicity of the extracts to a maximum extent. Most of the parts of *E. ferox* are highly consumed by the people of Manipur, India[15] and Manipuri people of Cachar District, Assam. While, tender leaves of other four plants are consumed by Khasi and Naga tribes of Machkhal, Binnakandi, Ramnagar and few other tribal pockets of Cachar district.

The fruits of *E. ferox* can be preserved for a longer period without any preservative agent,[14] which may be attributed to the compounds with antimicrobial property. Chakma tribe in hill tracts districts of Bangladesh uses leaf paste of *S. pulcherrima* to treat boils and fever blisters and fresh leaf extract as eye drop.[16] *Garcinia* species were reported to be good source of biologically active substances.[17,18] Phytochemical screening indicated dominance of phenolic compounds in *Garcinia* species.[19] However no information is available on *G. lancifolia* in respect to its antimicrobial activity as well as its chemical constituents. The leaf sap of *Gnetum gnemon* is used to cure an eye complication.[20] Extracts from fruits and seeds of *Gnetum* possess antibacterial and antioxidant activity and also exert various physiological (pharmacological) activities and hence proposed to be used as an active ingredient of food, nutritional supplement, medicine, cosmetic etc.[21] The ethnomedicinal informations are shown in Table 1. In spite of having several medicinal properties of these plants, so far anticandidial activity has not been reported. Hence, the present study was undertaken to test the efficacy of these plant species against *Candida albicans*.

MATERIALS AND METHODS

Plant materials and extraction

Fresh plant samples were collected from *Arun Punjee* (*Punjee* means Tribal Village) of Machkhal, Cachar district, Assam during the month of April. The taxonomic identities of the plants were authenticated at Botanical Survey of India, Eastern Regional Centre, Shillong, Meghalaya, India. The herbaria were deposited at the herbarium repository of Defence Research Laboratory, Tezpur, Assam. Extract was prepared by soaking powdered air dried plant sample in methanol for 7 days.[23] The solution was filtered, concentrated under reduced pressure at 40°C and lyophilized. The extractive values were recorded (Table 2) and kept in -20°C till further use. Stock solution of each extract (2×10^5 µg/ml, w/v) was

Table 1. Ethnomedicinal information of the test plants.

Species Family	Local name (Community)	Part used	Uses
C. colebrookianum Verbenaceae	*Jhurkhthang* (khasi)	Leaves	High blood pressure[34]
G. gnemon Gnetaceae	*Shayang* (khasi)	Leaves	Athletes foot[22], eye complication[20]
S. pulcherrima Urticaceae	*Khajathshear* (Khasi)	Leaves	Boils and fever blisters, itching of eyes[16], to damage tape worm egg in pork[22]
G. lancifolia Clusiaceae	*Shasuit* (Khasi)	Leaves	Headache[22]
E. ferox Nymphaeaceae	*Thangjing* (Manipuri)	Seeds	Digestive disotders, diarrheaetc.[14] Eye ailment (personal interrogation)

Table 2. Anticandidial activity and total phenolic content (TPC) of plant extracts.

Plant species	EV (%) w/w	Conc. (µg/ml)									TPC[a]	MIC (µg/ml)
		2×10^5			1×10^5			5×10^4				
		ZOI	AI	I%	ZOI	AI	I%	ZOI	AI	I%		($\times 10^4$)
C. colebrookianum	23.35	17	0.57	21.3	11	0.36	13.8	9	0.30	11.3	0.0429	2.5
G. gnemon	23.02	18	0.60	22.5	12	0.40	15.0	10	0.33	12.5	0.0452	2.5
S. pulcherrima	19.89	21	0.70	26.3	16	0.53	20.0	13	0.43	16.3	0.33	1.25
G. lancifolia	20.40	20	0.67	25.0	14	0.47	17.5	12	0.40	15.0	0.0443	2.5
E. ferox	17.80	25	0.83	31.3	24	0.80	30.0	20	0.67	25.0	0.456	1.25
Correlation coefficient			0.899			0.908			0.887			–0.774
Clotrimazole (100 µg/ml)		ZOI: 17mm										
DMSO (100%)		No zone of inhibition										

EV: Extractive value, ZOI: Inhibition zone (diameter in mm), AI: Activity index, I%: Inhibition percentage, MIC: Minimum inhibitory concentration.

[a] In mg Gallic Acid equivalent (GAE)/mg of dried extract.

prepared in dimethyl sulfoxide (DMSO). From the stock, the test extracts of desired concentrations were prepared in DMSO and sterilized using millipore filter (0.22 µm pore size).

Candida albicans and inoculum preparation

C. albicans (MTCC 3018) was cultured on sabouraud dextrose agar (SDA) slants at 28±2°C for 48 hours and stored at 4°C. Broth inoculum was prepared using sabouraud dextrose broth (SDB) and incubated at 28±2°C for 48 hours and final inoculum concentration of 1×10^8 CFU ml^{-1} was prepared.[8]

Anticandidial assay

The activity of the plant extracts against the test organism was evaluated by agar well diffusion assay with some modification.[24] Inoculum of 150 µl was swabbed on a SDA plate, a well of 7 mm diameter was made in each agar plate and loaded with 200 µl of the respective test extracts (2×10^5, 1×10^5 and 5×10^4 µg/ml) The SDA plates were incubated at 28±2°C for 48 h. Anticandidial activity of each extract was expressed in terms of diameter of inhibition zone (mm) exhibited by the extracts. DMSO (100%) was used as negative control while clotrimazole (100 µg/ml)[25] was used as positive control. Each experiment was replicated thrice and repeated twice. The activity index of the extracts and percent inhibition were calculated as follows.

$$\text{Activity index} = \frac{\text{Zone of inhibition by extract}}{\text{Zone of inhibition by standard antimicrobial agent}} \quad [26]$$

$$\% \text{ Inhibition} = \frac{\text{Zone of inhibition (mm)}}{*\text{Control}} \times 100 \quad [27]$$

*Growth zone is equal to plate diameter i.e., 80 mm, as growth occurs all over the agar plate.

Determination of MIC

Minimum inhibitory concentration (MIC) values of the extracts were determined by broth microdilution method[28] with some modifications. Stock solution of each sample was serially diluted in 96-well microtiter plate with RPMI 1640 (Rosewell Park Memorial Institute, Himedia) to obtain a concentration ranging from 2.5×10^4 to 1.56×10^4 µg/ml. Inoculum density of 1×10^8 CFU/ml^{-1} approximately was adjusted in each well and incubated at 28 ± 2°C for 48 h. Clotrimazole was used as standard. The MIC of extract was interpreted as the lowest concentration, at which no visible growth was seen. Each experiment was performed in triplicate and repeated twice.

Statistical analysis

Statistical analysis was done with SPSS, version 17.0.

RESULTS AND DISCUSSION

Previous workers showed antifungal activity of *Zingiber officinale* and *Juglans cinera* against a diverse group of human pathogenic fungi including strains that are highly resistant to amphotericin-B and ketoconazole and interestingly, in many cases, the medicinal plants were found more effective than the commercial antimicrobial drugs.[4] Similarly, Sehgal et al. (2005) reported the higher inhibitory action of petroleum ether and methanol extract of latex from *Calotropis procera* against *Candida albicans*, as compared to griseofulvin.[5] Even crude ethanol extracts of *Acacia nilotica, Cinnamum zeylanicum* and *Syzygium aromaticum* showed good activity against multidrug resistant strains, isolated from nosocomial and community acquired infections, where modern antibiotic therapy has failed. The antimicrobial potency of these plants is believed to be due to tannins, saponins, phenolic compounds, essential oils and flavonoids.[6] Several other studies demonstrated that *Candida* species are either highly resistant or less sensitive towards plant extracts as compared to moulds and other pathogenic microbes.[7–10]

Anticandidial activity and MIC of the plant extracts

In the initial screening, all the plants showed anticandidial activity, as evidenced by clear zone of inhibition ranged between 9 and 25 mm within the concentrations tested. In DMSO treated plate no zone was observed while clotrimazole (100 µg/ml) produced zone of inhibition of 17 mm. Activity of clotrimazole at very low concentration, as compared to the plant extracts may be attributed to its pure nature. On subsequent test for determination of MIC value, varying degree of sensitivity of the yeast towards each of the extracts (2.5×10^4 to 1.56×10^4 µg/ml) was recorded. In this study, *E. ferox* (seed) and *S. pulcherrima* (leaf) were found to be the most active with MIC value of 1.25×10^4 µg/ml. The MIC values of the remaining extracts were 2.5×10^4 µg/ml (Table 2). Since the crude extracts are mixture of multiple components, both active and non active, they are considered as effective even the MICs were high. Similar results were also recorded earlier.[29–30]

In our previous study, varied total phenolic contents of the extracts (0.456–0.0429 mg GAE/mg dry weight of

extract) was recorded (Table 2), with highest phenolic content in *E. ferox* seed extract (0.456 mg GAE/mg DW) followed by *S. pulcherimma* leaf extract (0.33 mg GAE/mg DW).[22] The strong correlation of total phenolic content with anticandidial activity might be due to phenolic compounds, which are responsible for antioxidant action. Significant antioxidant activity of *E. ferox* was reported earlier.[31] The observed anticandidial activity of the plant extracts might be related to the high level of phenolic compounds along with other phytochemicals.[22,32] Earlier reports projected a positive correlation of phenolic compounds with antimicrobial activity.[33] However, the two parameters may not always correlate.[23] Total phenolic content estimated in the extracts in our earlier study showed strong negative correlation with the MIC of the extracts (correlation coefficient is –0.774, which signifies strong correlation between two variables).

The reported biological activity of the plants as well as their extensive use in traditional practice suggests that these plants have the potential to be a very useful antimicrobial agent particularly against *Candida albicans*. Although medicinal property of *E. ferox* and its traditional uses for treatment of various ailments have been reported earlier, information on scientific evaluation for its antifungal activity has not been done yet.[14,31] On the other hand, the significant activity of *S. pulcherrima* and *G. lancifolia* against *C. albicans* is probably the first report.

CONCLUSIONS

The results highlighted the possible use of *E. ferox*, *S. pulcherrima* and *G. lancifolia* as therapeutic agents against *Candida albicans*. However toxicity and clinical studies are required to validate the use of these medicinal plants in therapeutics. The anticandidial activity of *S. pulcherrima* and *G. lancifolia* is the first time report, to the best of our knowledge. Further studies are going on *in vivo* evaluation on animal model, isolation and characterization of bioactive compounds from these plants.

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Evaluation of anti-helmintic and wound healing potential of *Saraca asoca* (Roxb) bark

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ABSTRACT

Introduction: The present work was done in search of traditional cure for live stock diseases and injuries occur to the body. The present study was aimed to evaluate wound healing potential of *Saraca asoca* bark in the form of simple ointment using two types of wound models in rats as incision wound and excision wound models and also evaluated for anti-helmintic activity. **Materials and Methods:** The methanolic extract of *Saraca asoca* Roxb. was evaluated for its anti-helimintic and wound healing activity at doses of 50 mg/ml and 100 mg/ml for anti-helmintic activity and 1% w/w, 4% w/w ointments in the case of wound healing activity. Albendazole 10 mg/ml is used as standard for antihelmintic activity and nitrofurazone 0.2% w/w ointment served as standard in the wound healing model. The parameters studied are time taken for paralysis and death of worms for anti-helmintic activity, tensile strength in incision wound model, percentage wound closure and period of epithelialisation in excision wound model. **Results:** Highest dose of this extract was found to possess good anti-helmintic activity along with significant wound healing activity. This was evident by decrease in time taken for paralysis and death; significant increase in tensile strength and decrease in period of epithelialization; increase in wound contraction when compared to control. **Discussion:** The progressive results are may be due to depletion of glycogen or β-tubulin inhibition in helmentic worms and increase in the viability and strength of collagen fibers in rats by the presence of tannins, flavonoids and alkaloids.

Keywords: *Saraca asoca,* wound healing activity, anti-helmintic activity.

INTRODUCTION

Helimintic infections are one of the common infections affecting a large proportion of world's population. Helminths can live in humans and animals, and are usually transmitted through contaminated food, water, faeces, and unwashed hands or contact with a contaminated object. It is estimated that approximately one-third of the almost three billion people that live on less than two US dollars per day in developing regions of sub-Saharan Africa, Asia and the America are infected with one or more helminth.[1] Adding to the global morbidity that results from human helminth infections are the observations that they have both direct and indirect effects on malaria and HIV/AIDS in developing countries. In Sub-Saharan Africa and elsewhere, helminthiases are frequently coendemic with malaria and HIV/AIDS. [2] Mostly school aged children and pre school children tend to curb the excessive number of intestinal worms and schistosomes and as a result experience diminished growth, impaired memory.[3] Despite the remarkable success of mass drug administration, gastro intestinal helminthes quickly develops resistance towards currently available drugs.[4] Thus, research on new drugs is clearly needed for the treatment of helminthes. Therefore, the present study was carried out for antihelmintic activity. Wound is a break in the normal tissue integrity. It can be caused by either physical, chemical, thermal and microbial agents. Wounds, particularly among the elderly population, can show delayed or disturbed healing; however, delayed or disturbed healing is also evident in patients with comorbidities such as

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diabetes, atherosclerosis, venous/arterial insufficiency, reduced mobility due to chronic infirmity and hypercholesterolemia. To heal a wound, the body undertakes a series of actions collectively known as the wound healing process. The process of wound healing consists of integrated cellular and biochemical events leading to reestablishment of structural and functional integrity with regain of strength of injured tissue. Following injury, an inflammatory response occurs and the cells below the dermis (the deepest skin layer) begin to increase collagen (connective tissue) production. Later, the epithelial tissue (the outer skin layer) is regenerated. There are three stages in the process of wound healing: inflammation, proliferation, and remodeling. The proliferative phase is characterized by angiogenesis, collagen deposition, epithelialisation and wound contraction. Angiogenesis involves formation of new blood vessels from endothelial cells. In fibroplasia and granulation tissue formation, fibroblasts exert collagen and fibronectin to build a new, provisional extracellular matrix. Subsequently, epithelial cells crawl across the wound bed to cover it and the wound is contracted by myofibroblasts, which grip the wound edges and undergo contraction using a mechanism similar to that in smooth muscle cells.[5] A number of drugs ranging from simple non-expensive analgesics to complex and expensive chemotherapeutic agents were available in the management of wound healing either positively or negatively.[6] Because of draw backs and unwanted effects of current drugs, approaches towards medicinal plants provides promising results in accelerating the wound healing process progressively in a normal manner. As no scientific data has been reported on anti-helmintic and wound healing activity of methanolic extract of *Saraca asoca* Roxb, the present study was done to evaluate the anti-helmintic potential and wound healing activity. *Saraca indica* or *Saraca asoca* is a small evergreen tree having height up to 10 meters. It occurs up to the altitude of 750 meters. Other names are sita-ashok, anganapriya, hemapushpa, madhupushpa, vanjula, vishoka, kankeli, vichitra.[7] Leaves are narrowly lanceolate, cork like at the base and with a shot pestistipules are intra-petiolar and completely united. The bark is dark brown or grey or almost black with warty surface. Stem bark is rough and uneven due to the presence of rounded or projecting lenticles. It is channeled, smooth with circular lenticles and traversely ridged, sometimes cracked. Flowers are fragnant, yellowish orange turning to scarlet, in short laterally placed corymbose, axillary panicles, bract small, deciduod, calyx petaloid. Seeds are 4–8, ellipsoid-oblong and compressed.[8] It is effective against a great number of gynecological disorders, ash of the plant is good in rheumatoid arthritis.[9] It is also used in leucorrhoea and in internal bleeding, haemorrhoids and haemorrhagic dysentery.[10] Alcoholic extract of bark shows significant anti-microbial activity.[11]

MATERIALS AND METHODS

The bark of *Saraca asoca* was collected locally and allowed for shade drying. The shade dried bark was crushed in to pieces and powdered. About 100 gm of powdered bark was extracted using soxhlet apparatus for 12 hrs. Alcohol removal was carried out under reduced pressure afforded a solid powder with a yield of 10%.

PHYTOCHEMICAL SCREENING

Methanolic extract was evaluated for the presence of various phytoconstituents by performing different phytochemical tests. The results showed the presence of tannins, flavanoids, saponins, anthraquinone, cardiac glycosides, steroids and carbohydrates which were reported in table 1.

EXPERIMENTAL ANIMALS

Anti-helmintic activity

The anti-helmintic activity was evaluated on adult Indian earthworm, 'Pheretima posthuma' as it has anatomical and physiological resemblance with the intestinal round worm parasites of human beings.[12–13]

Wound healing activity

Wound healing activity was done on male albino rats weighing about 150–200 gm. They were fed with food and water *ad libitum*. They were housed in polypropylene cages and maintained under standard conditions.

Acute dermal toxicity

The acute dermal toxicity study was carried out in adult female albino rats by 'fix dose' method of OECD

Table 1. Chemical tests.

S.No	Chemical test	MESA
1	Alkaloids	+
2	Carbohydrates	+
3	Proteins and aminoacids	–
4	Steroids and Terpenoids	+
5	Saponins	+
6	Glycosides	+
7	Phenolic compounds	+
8	Tannins	+

+ = present; – = absent.

(Organization for Economic Co-operation and Development) guideline No. 434. Extract from the bark of *Saraca asoca* was applied topically at dose level 2000 mg/kg.

DRUG FORMULATIONS

Anti-helmintic activity

50 mg/ml and 100 mg/ml concentration solutions were prepared for methanolic extract. The extract was initially dissolved in dimethyl sulfoxide (DMSO) and the solutions were prepared in normal saline.

Wound healing activity

The methanolic extract of *Saraca asoca* was formulated as 1% w/w and 4% w/w ointments. These ointments were prepared by incorporating 1 g and 4 g of the extract respectively into 100 g of simple ointment base. The standard drug used for wound healing activity was soframycin 1% w/w ointment. Ointments were applied once a day to experimental animals with wounds until they were cured.

STUDY PROTOCOL

Anti-helmintic activity

Each group consists of 6 earth worms.

Group 1 – served as control and released in to normal saline.

Group 2 – served as standard and released in to Albendazole 10 mg/ml.

Group 3 – served as treatment group and released in to 50 mg/ml solution of methanolic extract of *Saraca asoca.*

Group 4 – served as treatment group and released in to 100 mg/ml solution of methanolic extract of *Saraca asoca.*

Wound healing activity

For each model 4 groups were used. Each group consists of 5 animals.

Group 1 – served as control for incision wound model and received only simple ointment base.

Group 2 – served as standard for incision wound model and received 1% w/w Soframycin ointment.

Group 3 – served as treatment group for incision wound model and received 1% w/w ointment of MESA (methanolic extract of *Saraca Asoca*)

Group 4 – served as treatment group for incision wound model and received 4% w/w ointment of MESA.

Group 5 – served as control for excision wound model received only simple ointment base.

Group 6 – served as standard for excision wound model and received 1% w/w Soframycin ointment.

Group 7 – served as treatment group for excision wound model and received 1% w/w ointment of MESA.

Group 8 – served as treatment group for excision wound model and received 4% w/w ointment of MESA.

EXPERIMENTAL PROCEDURE FOR ANTI-HELMINTIC ACTIVITY

The anthelmintic activity was performed according to the method described by T. Ghosh[14] on adult Indian earthworm *Pheritima posthuma* as it has anatomical and physiological resemblance with the intestinal roundworm parasites of human beings. The earthworms in each group were released into 50 ml of desired formulation. Observations were made for the time taken to paralyze or death of individual worms. Paralysis was said to occur when the worms do not revive even in normal saline. Death was concluded when the worms lose their motility followed with fading away of their body color.

EXPERIMENTAL PROCEDURE FOR WOUND HEALING ACTIVITY

Incision wound model: On the depilated backs of the animals, two paravertebral incisions of 6 cm length were made cutting through the full thickness of the skin. Interrupted sutures, 1 cm apart, were placed to approximate the cut edges of the skin.[15] The sutures were removed on the 7th post wound day and skin breaking strength was measured on the 10th day by continuous water flow technique of Lee.[16]

Excision wound model: An excision wound was inflicted by cutting away 500 mm^2 full thickness of a predetermined area on the depilated back of the rat. Epithelialization period was noted as the number of days after wounding required for the scar to fall off leaving no raw

wound behind. Wound contraction rate was monitored by planimetric measurement of the wound area on alternate days. This was achieved by tracing the wound on a graph paper. Reduction in the wound area was expressed as percentage of the original wound size.[17]

Statistical analysis

The means of wound area measurement and wound breaking strength between groups at different time intervals were compared using one-way ANOVA, followed by students T–test. In all tests the criterion for statistical significance was $p<0.05$.

RESULTS AND DISCUSSION

The preliminary phytochemical screening of the methanolic extract of *Saraca* showed the presence of triterpinoids, carbohydrates, phenols, saponins, tannins and flavonoids (Table 1). The MESA (100 mg/ml) showed significant ($p<0.001$) decrease in paralysis time and significant ($p<0.001$) decrease in death time when compared to albendazole and MESA (50 mg/ml) treated animals (Table 2). In the current study, albendazole that was used as the standard shows its action by blocking glucose uptake and inhibition of polymerization of β-tubulin.[18] Therefore, antihelmintic activity of methanolic extract of *Saraca asoca* may be associated with the depletion of glycogen stores or by β-tubulin inhibition which prevents vesicular transport. Anti-helmintic activities are well documented by the existence of active compounds such as flavonoids, tanins, triterpinoids.[19–20] Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers by regenerating dermal and epidermal tissue, in a predictable fashion to repair the damage.[21] There are three phases in this process such as inflammatory, proliferative and remodeling phases.[22] In the process of inflammation bacteria are phagocytosed and removed.[23] The cardinal symptoms of this phase are pain, swelling, redness.[24] Proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization and wound contraction.[23] In the maturation and remodeling phase, collagen is remodeled and realigned along tension lines and cells that are no longer needed are removed by apoptosis. Infection of wound by microorganisms delays the wound healing. However, it can't be seen with *Sarca asoca* as it has antimicrobial activity.[25] In Incision wound model the 4% w/w ointment of MESA showed significant ($p<0.001$) increase in tensile strength when compared to control, standard and MESA 1% w/w treated animals. The 1% w/w ointment of MESA showed significant ($p<0.01$) increase in tensile strength when compared to control animals (Table 3). Increase in breaking strength of SA – ointment treated animals improved collagen migration by increased cross linking. In excision model of study the MESA 4% w/w showed significant ($p<0.01$) wound closure activity when compared to the control animals on 4^{th} day. The MESA 4% w/w showed significant ($p<0.001$) wound closure when compared to the control animals on 8^{th}, 12^{th} and 16^{th} day. The MESA 4% w/w showed significant ($p<0.001$) activity in wound closure when compared to the Soframycin 1% w/w treated animals on 4^{th} and 16^{th} day. The MESA 4% w/w showed significant ($p<0.01$), ($p<0.05$) activity in wound closure when compared to the Soframycin 1% w/w treated animals on 8^{th} and 12^{th} day respectively. The MESA 4% w/w showed significant ($p<0.05$), ($p<0.01$) activity in wound closure when compared to the MESA 1% w/w treated animals on 8^{th} and 16^{th} days respectively. The MESA 1% w/w showed significant ($p<0.001$) activity in wound closure when compared to the control animals on 8^{th} and 12^{th} day. The MESA 1% w/w showed significant ($p<0.01$) activity in wound closure when compared to the control animals on 16^{th} day (Table 4). The MESA 4% w/w showed significant ($p<0.001$) wound closure activity in 14 days when compared to the control (21 days) and standard animals (18 days). The MESA 1% w/w showed significant ($p<0.001$) wound closure activity in 16 days when compared to the control animals. Wound contraction is defined as the centripetal movement of the edges of a full thickness wound in order to promote seal of the defect.[26] The rate of wound contraction was less in control and standard groups when compared to SA – ointment treated animals. Granulation tissue formed

Table 2. Anthelmintic activity of MESA.

Serial No	Groups	Dose (mg/ml)	Time taken for paralysis (min)	Time taken for death (min)
1	Control	–	–	–
2	Albendazole	10	65.66±0.333	77.16±1.046
3	MESA	50	26.16±0.654	29.16±0.307
4	MESA	100	22.5±0.562*+	24.83±0.307*+

Values are expressed as mean ± SEM (n = 6)
*(p<0.001)vs standard group, + (p<0.001) vs MESA – 50 mg group

Table 3. Effect of MESA on tensile strength in incision wound model.

Groups	Tensile strength (gm ± SEM)
Control	139.2 ± 0.374
Soframycin1%	187.4± 1.887
MESA 1%	185 ± 2.530**
MESA 4%	204 ± 3.987***+xxx

Values are expressed as mean ± SEM (n = 5)
(p<0.001)vs control group, (p<0.01)vs control group, + (p<0.001) vs standard group, xxx (p<0.001) vs MESA – 1% group

Table 4. Effect of MESA on wound closure in excision wound model.

Groups	% of closure of excision wound area				Epithelialization in days
	Day 4	Day 8	Day 12	Day 16	
Control	29.4±2.159	56.8±0.663	73.8±2.354	85.2±1.772	21±0.774
Soframycin 1%	31.8±1.463	66.6±1.435***	84.2±1.393**	89.4±0.509**	18.6±0.244*
MESA1%	33.6±0.600	68±1.378***	86.6±1.288***	90±0.316**	16.6±0.509***
MESA 4%	38.4±1.631**+++	74.4±1.939***++x	91.8±0.734***+	99.2±1.114***+++xx	14.4±0.678***+++

Values are expressed as mean ± SEM (n = 5)
***(p<0.001)vs control group, ** (p<0.01)vs control group ,* (P<0.05) vs control group
+++ (p<0.001)vs standard group, + + (p<0.01) vs standard group, + (p<0.05) vs standard group
xx (p<0.01) vs MESA – 1% group, x (p<0.05) vs MESA– 1% group

in the final part of the proliferative phase is primarly composed of fibroblasts, collagen, and new small blood vessels. Collagen is a major component that strengthens extracellular tissue and is composed of the amino acid, hydroxyproline, used as a biomarker for tissue collagen.[27] Oxidative stress is an intrinsic sector of wound healing leading to depletion of enzymatic and nonenzymatic antioxidants. In acute as well as in chronic wounds, the enzymatic antioxidants decreases and leads to depletion of non enzymatic antioxidants due to high oxidative stress.[28–29] The wound healing activity of this medicinal plant is attributed to the active constituents present in it. Already *in vitro* antioxidant activity of stem bark of *Saraca asoca* was investigated,[30] by this it is capable of destroying free radicals and prevents extensive tissue damage. The presence of flavonoids in the plant extract may reduce the lipid peroxidation by preventing or slowing the onset of cell necrosis and improves vascularity.[31] Tannins[32] and triterpinoids[33] are known to magnify wound healing process. The plant *Portulaca oleracea* containing the tannins possesses wound healing activity as that of the *R. cordifolia*.[34] The gel of ethanolic extract of the plant *Vernonia scorpioides* possess wound healing action by promoting regeneration and organization of the new tissue due to the presence of tannins.[35] The embellin isolated from the ethanol extract of plant *Emblica officinalis* containing condensed tannins when formulated as a gel possess significant wound healing property[36] as that of gel prepared by *R. cordifolia* ethanol extract. These active constituents promote the process of wound healing by increasing the viability of collagen fibers, by increasing the strength of collagen fibres either by increasing the circulation or by preventing the cell damage or by promoting the DNA synthesis.[37]

CONCLUSION

The results of the present study revealed that the phytoconstituents tannins, flavonoids, triterpinoids are known to avail anthelmintic and wound healing activities. These phytoconstituents decreased the life time of earthworms and this might be by the β-tubulin interference. Increased cellular proliferation may be due to the mitogenic activity of the plant extract, which might have significantly contributed to wound healing process. The extract had prominent effects towards cellular proliferation, granulation tissue formation and epithelialisation and this is evident by early dermal and epidermal regeneration. So far, fatality and lethality results in patients with wounds because of infections, these herbal extracts prevent high risk of sepsis and further prevent the prolongation of inflammatory phase. In conclusion, the present study suggests that the methanolic extract of *Saraca asoca* exerts anti-helemintic and wound healing activity.

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