Chemical constituents of *Broussonetia luzonicus*

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ABSTRACT

The leaves of *Broussonetia luzonicus* afforded epitaraxerol (**1**), lupenone (**2**), squalene (**3**), β-carotene (**4**), vitamin K (**5**) and β-sitosterol (**6**), while the flowers yielded **2**, **6**, lupeol (**7**), betulin aldehyde fatty acid ester (**8**) and lupeol fatty acid ester (**9**). The compounds were isolated by silica gel chromatography and identified by NMR spectroscopy. Triterpenes **1**, **2** and **8** were tested for cytotoxicity using the MTT assay. They did not exhibit cytotoxic effect against a human cancer cell line colon carcinoma (HCT 116). Results of the antimicrobial tests on **1**, **2** and **8** indicated moderate antifungal activity against *C. albicans* and low antimicrobial activity against *T. mentagrophytes, A. niger, S. aureus*, *E. coli, P. aeruginosa,* and *B. subtilis*.

Keywords: *Broussonetia luzonicus*, Moraceae, triterpenes, antimicrobial

INTRODUCTION

Broussonetia luzonicus (Moraceae family), is an endemic tree which grows in thickets and forests throughout the Philippines.[1] The flowers and leaves are eaten as vegetables. There are no reported studies on the chemical constituents and biological activities of *B. luzonicus*. However, *B. papyrifera* and *B. kazinoki* which are congeners of the tree have been used as tonic in traditional Chinese medicines. The chemical components of *B. papyrifera* were shown to decrease the effects of Alzheimer's disease and exhibit anti-aging properties, as well as possess antityrosinase and antioxidant activities.[2] On the other hand, *B. kazinoki* was reported to exhibit cytotoxic properties.[3]

We report herein the isolation and identification of epitaraxerol (**1**), lupenone (**2**), squalene (**3**), *β*-carotene (**4**), vitamin K (**5**) and *β*-sitosterol (**6**) from the leaves; and **2**, **6**, lupeol (**7**), betulin aldehyde fatty acid ester (**8**) and lupeol fatty acid ester (**9**) from the flowers of *B. luzonicus* (Fig. 1). We also report the antimicrobial properties of **1**, **2** and **8**.

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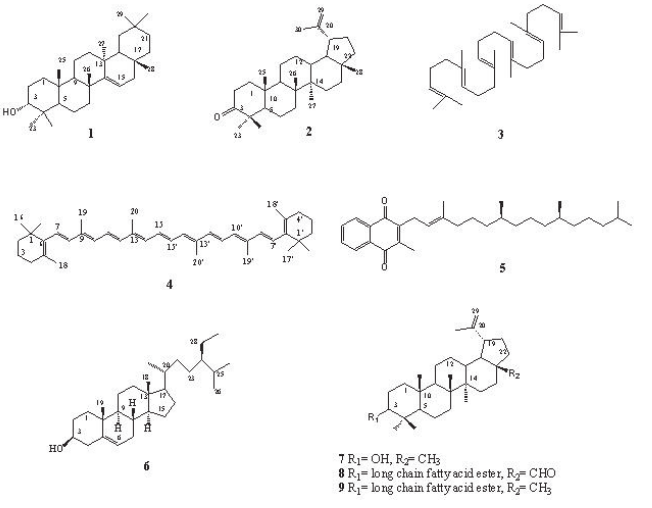

Figure 1. Chemical constituents of Broussonetia luzonicus leaves: epitaraxerol (1), lupenone (2), squalene (3). B-carotene (4), vitamin K (5) and B-sitosterol (6) and flowers: 2, 6, lupeol (7), betulin aldehyde fatty acid ester (8) and lupeol fatty acid ester (9).

MATERIALS AND METHODS

General Experimental Procedures

NMR spectra of **1-4**, **6** and **8** were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C-NMR spectra. NMR spectra of **5**, **7** and **9** were recorded on a Varian Unity Inova spectrometer in $CDCl_3$ at 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR spectra. Column chromatography was performed with silica gel (230–400 mesh). TLC was performed on aluminium plates coated by silica gel 60 F_{254}. The plates were visualized by spraying with vanillin-sulfuric acid, followed by warming.

Plant Material

The leaves of *Broussonetia luzonicus* were collected from the Bureau of Plant Industry (BPI), Department of Agriculture, Manila, Philippines, while the flowers were collected from Sinait, Ilocos Sur. The samples were authenticated by Josephine T. Garcia of the BPI.

Extraction and Isolation

The air-dried leaves (1.2 kg) of *B. luzonicus* were ground in an osterizer, soaked in dichloromethane (DCM) for three days, and then filtered. The filtrate was concentrated under vacuum to afford a crude extract (50 g) which was chromatographed in increasing proportions of acetone in DCM at 10% increment. The DCM fraction was rechromatographed (9x) in hexane to afford **3** (200 mg), **4** (150 mg) and **6** (80 mg). The 10–20% acetone in DCM fractions were rechromatographed (8x) in 2.5% EtOAc in hexane, followed by 5%, 10% and 15% EtOAc in hexane to afford **1** (3 mg), **2** (7 mg) and **5** (3 mg).

The air-dried flowers (262 g) of *B. luzonicus* were ground in an osterizer, soaked in DCM for three days, and then filtered. The filtrate was concentrated under vacuum to afford a crude extract (10 g) which was chromatographed in increasing proportions of acetone in DCM at 10% increment. The 30% to 50% acetone in DCM fractions were combined and rechromatographed (5x) in 2.5% EtOAc in hexane to afford **8** (6 mg) and **9** (10 mg). The more polar fractions were rechromatographed (2x) by gradient elution technique. The column was first eluted with 10% EtOAc in hexane to afford **2** (12 mg), followed by DCM:diethyl ether:acetonitrile (8:1:1) to afford **6** (25 mg). The 10% acetone fraction was rechromatographed (4x) in DCM to afford **7** (120 mg), after washing with acetone.

Epitaraxerol (**1**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 32.2 (C-1), 25.1 (C-2), 76.2 (C-3), 37.4 (C-4), 49.2 (C-5), 18.7 (C-6), 41.2 (C-7), 39.1 (C-8), 48.9 (C-9), 35.8 (C-10), 17.4 (C-11), 33.7 (C-12), 37.5 (C-13), 158.2 (C-14), 116.7 (C-15), 37.7 (C-16), 38.0 (C-17), 48.7 (C-18), 36.7 (C-19), 28.8 (C-20), 32.2 (C-21), 35.1 (C-22), 28.2 (C-23), 22.2 (C-24), 15.2 (C-25), 26.0 (C-26), 21.2 (C-27), 29.8 (C-28), 33.3 (C-29), 29.9 (C-30).

Lupenone (**2**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 39.6 (C-1), 34.1 (C-2), 218.2 (C-3), 47.3 (C-4), 54.9 (C-5), 19.7 (C-6), 33.5 (C-7), 40.8 (C-8), 49.8 (C-9), 36.9 (C-10), 21.5 (C-11), 25.1 (C-12), 37.5 (C-13), 42.9 (C-14), 27.4 (C-15), 35.5 (C-16), 43.0 (C-17), 48.2 (C-18), 47.9 (C-19), 150.8 (C-20), 29.8 (C-21), 40.6 (C-22), 26.6 (C-23), 21.0 (C-24), 15.8 (C-25), 16.0 (C-26), 14.5 (C-27), 18.0 (C-28), 109.4 (C-29), 19.3 (C-30).

Squalene (**3**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 25.7 (C-1), 131.3 (C-2), 124.3 (C-3), 26.7 (C-4), 39.7 (C-5), 134.9 (C-6), 124.3 (C-7), 26.7 (C-8), 39.7 (C-9), 134.9 (C-10), 124.3 (C-11), 28.3 (C-12), 17.7 (C-2'), 16.0 (C-6'), 16.0 (C-10').

β-Carotene (**4**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 34.3 (C-1, 1'), 39.6 (C-2, 2'), 19.3 (C-3, 3'), 33.1 (C-4, 4'), 129.3 (C-5, 5'), 137.9 (C-6, 6'), 126.6 (C-7, 7'), 137.8 (C-8, 8'), 136.0 (C-9, 9'), 132.4 (C-10, 10'), 130.0 (C-11, 11'), 137.2 (C-12, 12'), 136.5 (C-13, 13'), 130.8 (C-14, 14'), 125.0 (C-15, 15'), 29.7 (C-16, 16'), 29.0 (C-17, 17'), 21.8 (C-18, 18'), 12.82 (C-19, 19'), 12.76 (C-20, 20').

Vitamin K (**5**): ^{13}C NMR (125 MHz, $CDCl_3$): δ 12.7, 16.3, 19.70, 19.71, 22.6, 22.7, 24.4, 24.8, 25.3, 26.0, 28.0, 32.6, 32.8, 36.6, 37.3, 37.4, 39.4, 40.0, 118.8, 126.2, 126.3, 132.15, 132.19, 133.3, 134.0, 138.0, 143.4, 146.2, 184.6, 185.5.

β-Sitosterol (**6**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 37.2 (C-1), 31.6 (C-2), 71.8 (C-3), 42.3 (C-4), 140.7 (C-5), 121.7 (C-6), 31.9 (C-7), 31.9 (C-8), 50.1 (C-9), 36.1 (C-10), 21.1 (C-11), 39.8 (C-12), 42.3 (C-13), 56.7 (C-14), 24.3 (C-15), 28.2 (C-16), 56.0 (C-17), 11.8 (C-18), 19.4 (C-19), 36.5 (C-20), 19.0 (C-21), 33.9 (C-22), 29.1 (C-23), 45.8 (C-24), 26.0 (C-25), 18.8 (C-26), 19.8 (C-27), 23.1 (C-28), 12.0 (C-29).

Lupeol (7): ^{13}C NMR (125 MHz, $CDCl_3$): δ 38.9 (C-1), 27.4 (C-2), 79.0 (C-3), 38.7 (C-4), 55.3 (C-5), 18.0 (C-6), 34.3 (C-7), 40.8 (C-8), 50.4 (C-9), 37.2 (C-10), 20.9 (C-11), 25.1 (C-12), 38.1 (C-13), 42.8 (C-14), 27.4 (C-15), 35.6 (C-16), 48.0 (C-17), 48.3 (C-18), 48.0 (C-19), 151.0 (C-20), 29.8 (C-21), 40.0 (C-22), 28.0 (C-23), 15.4 (C-24), 16.1 (C-25), 16.0 (C-26), 14.5 (C-27), 18.3 (C-28), 109.3 (C-29), 19.3 (C-30).

Betulin aldehyde fatty acid ester (**8**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 38.7 (C-1), 26.7 (C-2), 80.5 (C-3), 39.7 (C-4), 55.4 (C-5), 18.1 (C-6), 34.9 (C-7), 40.8 (C-8), 50.4 (C-9),

37.8 (C-10), 20.7 (C-11), 25.5 (C-12), 39.7 (C-13), 42.5 (C-14), 26.7 (C-15), 29.2 (C-16), 59.3 (C-17), 47.5 (C-18), 48.0 (C-19), 149.7 (C-20), 29.5 (C-21), 33.2 (C-22), 28.8 (C-23), 16.0 (C-24), 16.2 (C-25), 16.5 (C-26), 14.2 (C-27), 206.7 (C-28), 110.2 (C-29), 19.0 (C-30), 173.7 (C-1'), 34.9 (C-2'), 31.9 (C-3'), 22.7, 25.2, 29.2-29.8 (CH_2)$_n$, 14.1 (CH_3).

Lupeol fatty acid ester (**9**): ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.77, 0.82, 0.84, 0.86, 0.92, 1.02 and 1.66 (each 3H, s, H-23, 24, 25, 26, 27, 28, and 30), 2.35 (1H, m, H-19), 4.46 (1H, dd, *J* = 4.5, 11.0, H-3), 4.55 (1H, brs, H-29b), 4.73 (1H, brs, H-29a), fatty acid: 1.21-1.35 (br s, CH_2), 2.30 (2H, t, *J* = 7.0 Hz, H-2'), 1.60 (2H, H-3'), 0.86 (CH_3, t, *J* = 6.5 Hz).

BIOASSAYS

Cytotoxicity Tests

Four milligrams each of **1**, **2** and **8** were dissolved in 1 milliliter of DMSO to make 4 mg/mL solutions. Compounds **1**, **2** and **8** were tested for cytotoxic activity against a human cancer cell line colon carcinoma (HCT 116) at the Institute of Biology, University of the Philippines, Diliman, Quezon City. Doxorubicin was used as the positive control, while DMSO was used as the negative control. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay reported in the literature was employed.[4,5]

Antimicrobial Tests

The microorganisms used: *Pseudomonas aeruginosa* (UPCC 1244), *Bacillus subtilis* (UPCC 1149), *Escherichia coli* (UPCC 1195), *Staphylococcus aureus* (UPCC 1143), *Candida albicans* (UPCC 2168), *Trichophyton mentagrophytes* (UPCC 4193) and *Aspergillus niger* (UPCC 3701) were obtained from the University of the Philippines Culture Collection (UPCC). The test compound was dissolved in 95% ethanol. The positive control for the bacteria and fungi, respectively are chloramphenicol and Canesten which contains 1% chlotrimazole. The antimicrobial assay procedure reported in the literature[6] was employed. The clearing zone was measured in millimeters, and the average diameter of the clearing zones was calculated. The activity index was computed by subtracting the diameter of the well from the diameter of the clearing zones divided by the diameter of the well.

RESULTS AND DISCUSSION

The structures of **1**, **2**, **5**, and **7** were elucidated by extensive 1D and 2D NMR spectroscopy and confirmed by comparison of their ^{13}C NMR data with those reported in the literature for epitaraxerol,[7] lupenone,[8] vitamin K[9] and lupeol,[10] respectively. The structures of **3**, **4** and **6** were confirmed by comparison of their ^{13}C NMR data with those reported in the literature for squalene,[11] β-carotene[12] and β-sitosterol,[12] respectively. The structure of **8** was elucidated by extensive 1D and 2D NMR spectroscopy and confirmed by comparison of its ^{13}C NMR data with those of betulin aldehyde for the triterpene part[13] and fatty acid esters of 16-hydroxycycloartenol for the fatty acid part.[14] The structure of **9** was deduced by comparison of its ^{1}H NMR data with those of lupeol fatty acid ester.[15]

Since a congener of the tree exhibited cytotoxic properties,[3] **1**, **2** and **8** were tested for cytotoxicity against a human cancer cell line colon carcinoma (HCT 116) by the MTT assay. These compounds did not show linear interpolation with HCT 116, thus IC_{50} could not be computed. This implied that **1**, **2** and **8** did not exhibit cytotoxic effect against this cell line.

Compounds **1**, **2** and **8** were further tested for antimicrobial activity against seven microorganisms (Table 1). Results of the study indicated that these compounds exhibited moderate antifungal activity against *C. albicans* with activity index (AI) of 0.3, 0.4, and 0.4, respectively. They showed low antifungal activity against *T. mentagrophytes* (AI = 0.2, 0.3 and 0.4, respectively) and *A. niger* (AI = 0.1, 0.2 and 0.2, respectively). They also exhibited low antibacterial activity against *S. aureus* (AI = 0.2, 0.3, 0.3, respectively), *E. coli* (AI = 0.2, 0.2 and 0.1, respectively), *P. aeruginosa* (AI = 0.2, 0.2 and 0.2, respectively), and partial inhibition of *B. subtilis* (AI = 0.3, 0.5 and 0.5, respectively).

Although there are no known medicinal properties of *B. luzonicus*, results of the study implied that **1**, **2** and **8** exhibit antimicrobial potentials. The compounds isolated from *B. luzonicus* have shown biological activities. Squalene exhibited chemopreventive activity against colon carcinogenesis and possessed antioxidant properties.[16,17] Lupeol showed antiurolithiatic and diuretic activities, prevented the vesical calculi and reduced the stone size in the rats.[18] Long-chain fatty acid esters of lupeol demonstrated an antiplasmodial activity,[15] while β-sitosterol induced apoptosis in human tumors for colon and breast cancers.[19] Lupenone and lupeol exhibited antimicrobial, antiviral, anticancer, and anti-inflammatory activities.[20] Vitamin K is a factor required for normal blood coagulation and it reduced undercarboxylated osteocalcin and improved the bone turnover profile.[21]

Table 1. Antimicrobial Activity of Compounds 1, 2 and 8.

Microorganism	Compounds	Clearing Zone,[c] mm	Activity Index (AI)
Pseudomonas aeruginosa	1	12	0.2
	2	12	0.2
	8	12	0.2
	Chloramphenicol[c]	14	1.3
Staphylococcus aureus	1	12	0.2
	2	13	0.3
	8	13	0.3
	Chloramphenicol[c]	23	2.8
Candida albicans	1	(13)[c]	(0.3)[c]
	2	(15)[c]	(0.5)[c]
	Chloramphenicol[c]		
	1	13	0.3
	2	14	0.4
	8	14	0.4
	Canesten, 0.2 g[c]	18	0.8
T. mentagrophytes	1	12	0.2
	2	813	0.3
	8	14	0.4
	Canesten, 0.2 g[c]	55	4.5
Aspergillus niger	1	11	0.1
	2	12	0.2
	Canesten, 0.2 g[c]	23	1.3

[a]Average of 3 trials, [b]Partial inhibition of growth of test organism, [c]Chloramphenicol disc - 6 mm diameter, [d]Contains 1% Chlotrimazole

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Chemical examination of leaves of *Cinnamomum malabatrum* (Burm. f.) Blume sold as *Tamalapatra*

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ABSTRACT

Introduction: Leaves of *Cinnamomum tamala* (Lauraceae) commonly known as bay leaf or *tamalapatra* is a highly reputed commodity in drug and spice trade. Its adulteration with other leaf species belonging to genus *Cinnamomum* is found to be a common practice. While macro-microscopy is of great help in establishing the botanical identity of such doubtful samples, physico-chemical parameters in combination with TLC fingerprints and phytochemical characterization is a valuable tool to establish chemical identity. **Methods:** Survey of south Indian crude drug markets was done to find if in place of *C. tamala* some other leaves of *Cinnamomum* species are sold. Fresh leaves of various *Cinnamomum* species, including *C. tamala*, growing in south India were collected and studied comparatively. Leaves sold in markets of S. India under the name of *tamalapatra* were collected and subjected for detailed physico-chemical, HPTLC and GC-MS analysis. **Results:** Leaves of *C. malabatrum* showed marked distinction in physico-chemical and volatile oil composition which will serve as markers to differentiate it from *C. tamala*; the official source of *tamalapatra*. Though physico-chemical constants will serve the purpose of standardization, volatile oil composition was found to be a diagnostic test for the differentiation of *C. malabatrum* from *C. tamala*. **Conclusion:** Chemical identity of *C. malabatrum* was established in comparison with the official drug. Further biological studies may be confirmative in designating it as a valid substitute or adulterant for *C. tamala*.

Keywords: bay leaf, GC-MS, HPTLC, *tamalapatra*.

INTRODUCTION

Adulteration is a major problem one comes across while assessing the identity and quality of many herbal drugs.[1] For establishing their correct identity they are needed to be examined thoroughly. Leaves of the genus *Cinnamomum* are found to exhibit a number of similar macro-microscopic characters and hence most likely to be used as adulterant for official drug. *Cinnamomum* is a genus of the family Lauraceae having humpty number of examples related to the issue of adulteration with closely knit species.[2] Survey of the crude drug market of South India revealed that leaves of *Cinnamomum malabatrum* are sold commonly in place of the official drug *C. tamala*. As *tamalapatra* is an important commodity in Ayurvedic medicine,[3] it was thought worthwhile to undertake this investigation.

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MATERIALS AND METHODS

Plant materials

Dried leaves sold in the name of *tamalapatra* were collected from markets of various places in India. The authentic samples of leaves of *Cinnamomum malabatrum* were collected from Tropical Botanical Garden and Research Institute, Palode, Trivandrum, Kerala. Leaves were shade dried, powdered and stored in air tight containers for further chemical evaluation. Collected leaves of different species of *Cinnamomum* were identified and authenticated with the help of available literature.[4-6]

Instrumentation and techniques

Physico-chemical characters were performed as per WHO guidelines and volatile oil of the sample of

C. malabatrum and *C. tamala* were distilled using Clavenger's apparatus.[7] Preliminary phyto-chemical tests were done as per procedure. TLC characterization of the *n*-hexane extract was done as per standard procedure.[8-9] Ten and twenty µl of the extracts were applied on a precoated silica gel F254 on aluminum plates to a band width of 8 mm using Linomat 5 TLC applicator. The plate was developed in benzene : ethyl acetate (9.9 : 0.1) and the developed plates were visualized and scanned under UV (254 and 366 nm) and after derivatisation in vanillin-sulphuric acid spray reagent at 620 nm. R_f, colour of the spots, densitometric scan and superimposability of densitogram were recorded.[8] GC-MS of volatile oil of *C. malabatrum* was carried out using Shimadzu gas chromatography unit with a SE-30 10% Chromosorb-W packed stainless steel column (2 m × 2 mm). Oven programme: 60°C (5 min), 60–260°C (5°C/min), 260°C (10 min); carrier gas: nitrogen, flow rate 40 ml/min; injector temperature 240°C; detector temperature 240°C. Individual components were identified by database of mass spectra matching with published literature available in the digital libraries like NIST and WILEY.

RESULTS

Results obtained for the physico-chemical tests are presented in Table 1. The alcoholic extracts were tested positive for sugars, terpenes and tannins. R_f values of the spots and their colour on TLC of *n*-hexane extract is tabulated in Table 2. TLC photodocumentation of *n*-hexane extract is shown in Figure 1. HPTLC densitometric scan of *n*-hexane extract of *C. tamala* and *C. malabatrum* at UV 366 nm and after derivatisation with vanillin-sulphuric acid at 620 nm are shown in Figure 2. The GLC chromatogram for the volatile oil of *C. malabatrum* is presented in Figure 3. Compounds detected by GC-MS of the volatile oil in *C. malabatrum* and the percentages of the compounds detected are tabulated in Table 3.

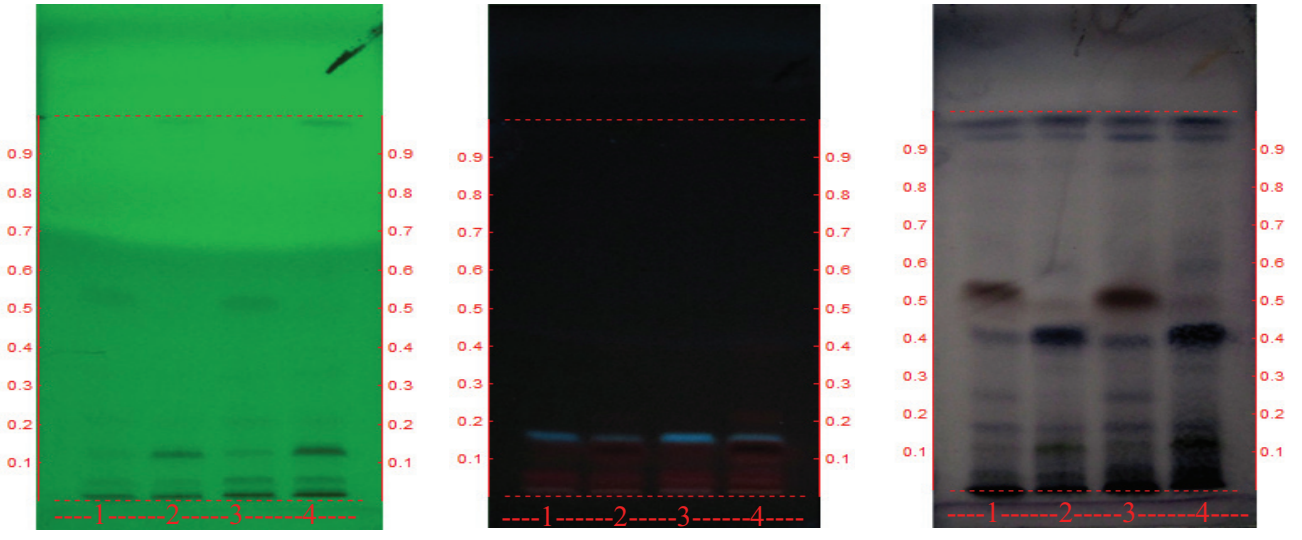
Table 1. Comparative physico-chemical characters *C. tamala* and *C. malabatrum*.

Parameters	*C. tamala*	*C. malabatrum*
Loss on drying at 105°C % w/w	9.63	9.27
pH of water soluble extractive	6.9	7.72
Total ash % w/w	2.78	2.92
Acid insoluble ash % w/w	0.53	0.34
Water soluble ash % w/w	1.31	0.45
n-Hexane soluble extractive	1.91	0.65
Alcohol soluble extractive % w/w	2.94	2.15
Water soluble extractive % w/w	10.29	6.62
Swelling factor ml	21	24
Volatile oil % v/w	0.70	0.55

Table 2. R_f values of *n*-hexane extract of *C. tamala* and *C. malabatarum*.

UV 254		UV 366		Post-derivatisation	
Ct	*Cm*	*Ct*	*Cm*	*Ct*	*Cm*
–	–	0.03 F pink	–	–	–
0.05 Green	0.05 Green	0.05 F pink	0.05 F pink	0.05 Blue	0.05 Blue
–	–	0.08 F pink	–	–	–
–	–	–	0.11 F pink	–	–
0.13 Green	0.13 Green	–	–	0.14 Brown	0.14 Brown
–	–	0.16 F blue	0.16 F blue	–	–
–	–	–	–	0.19 Blue	0.19 Blue
0.22 Green	0.22 Green	–	0.22 F pink	–	–
–	–	–	–	0.26 Blue	–
–	0.33 Green	–	0.33 F pink	–	–
–	–	0.41 F pink	0.41 F pink	0.41 Blue	0.41 D blue
0.52 Green	–	–	–	0.52 Brown	–
–	–	–	–	–	0.61 L blue
–	–	–	–	–	0.66 L blue
–	–	–	–	0.86 L blue	–
–	–	–	–	0.95 Blue	0.95 Blue

D – dark; F – fluorescent; L – light

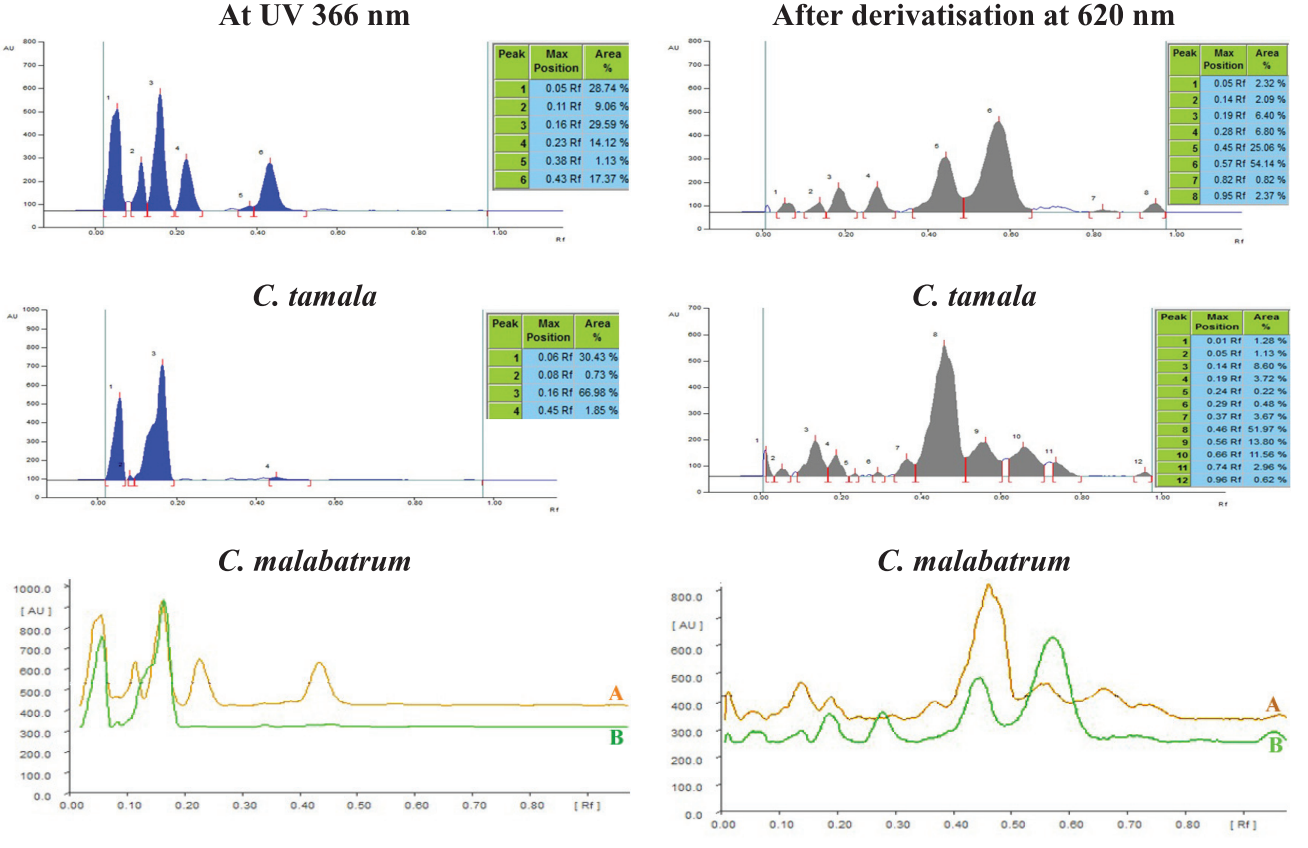


At UV 254 nm | At UV 366 nm | After derivatisation

Track 1 – n-hexane extract of C. *tamala* (10 µl)
Track 2 – n-hexane extract of C. *malabatrum* (10 µl)
Track 3 – n-hexane extract of C. *tamala* (20 µl)
Track 4 – n-hexane extract of C. *malabatrum* (20 µl)

Solvent system – Benzene : Ethyl acetate (9.9 : 0. 1)

Figure 1. TLC photodocumentation of *n*-hexane extract of *C. tamala* and *C. malabatarum*.



A – *C. tamala*; B – *C. malabatrum*

Figure 2. HPTLC densitometric scan of *n*-hexane extract.

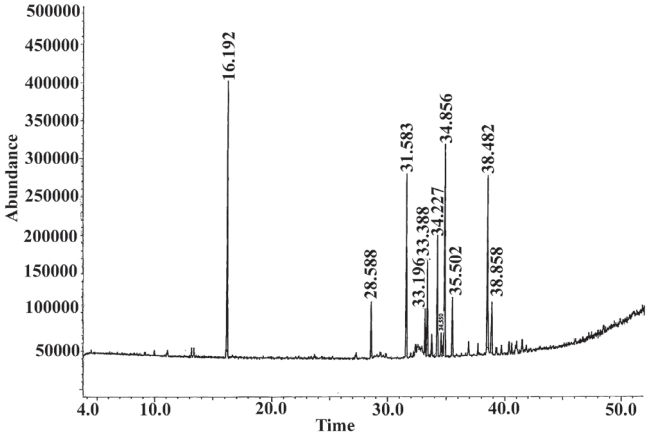


Figure 3. GLC chromatogram for the volatile oil of *C. malabatrum*.

Table 3. Compounds detected by GC-MS of *C. malabatrum* leaf volatile oil.

Compounds	RT	Area %
Linalool	16.192	17.80
Eugenol	28.588	3.70
Trans-caryophyllene	31.583	14.38
α-Humulene	33.196	2.96
Alloaromadendrene	33.388	7.42
Germacrene	34.227	9.50
Ledene	34.550	2.84
Bicyclogermacrene	34.856	18.23
δ-Cadinene	35.502	4.33
Spathulenol	38.482	13.88
Epiglobulol	38.858	4.95

RT – retention time (min)

DISCUSSION

On account of morphological similarity, characteristic fragrance and wider distribution in Western Ghats, leaves of *C. malabatrum* are sold in crude drug markets. For flavouring various edibles and for use in many Ayurvedic and Siddha formulations, this market substituent is found under the name of *tamalapatra*. Physico-chemical composition of the two samples showed difference in values for water soluble ash, *n*-hexane soluble matter, water soluble matter, swelling factor and percentage of volatile oil. Water soluble ash was 1.31% for *C. tamala* while the value was 0.45% for *C. malabatrum*. *n*-hexane soluble matter of *C. tamala* was 1.91% but it was 0.65% for *C. malabatrum*. Swelling factor is found to be of diagnostic importance to differentiate the two, *C. malabatrum* showed more mucilage content (24 ml) when compared to *C. tamala* (21 ml). Percentage of volatile oil in the sample examined was almost comparable to the *C. tamala* but is less by 0.25% v/w in *C. malabatrum*.

When the plate was viewed under 254 nm, *C. tamala* and *C. malabatrum* showed same number of spots. Spot with R_f values 0.52 was present only in *C. tamala* and 0.33 was present only in *C. malabatrum*. Under 366 nm, *C. malabatrum* showed 6 spots, whereas *C. tamala* showed only 5 spots. Spots with R_f value 0.03 and 0.08, both fluorescent pink were present only in *C. tamala* and 0.11, 0.22 and 0.33 (all fluorescent pink) were observed only in *C. malabatrum*. After derivatisation with vanillin sulphuric acid spray reagent, *C. tamala* showed 8 spots whereas *C. malabatrum* showed only 7 spots. Spots with R_f values 0.26 (blue), 0.52 (brown) and 0.86 (light blue) were present only in *C. tamala* and 0.61 (light blue) and 0.66 (light blue) were observed in *C. malabatrum* only.

Compounds such as eugenol, linalool, α-pinene, camphene, β-pinene, benzaldehyde, myrcene, limonene, p-cymene, benzyl acetate, α-terpineol, cinnamaldehyde, geraniol, linalool acetate and benzyl cinnamate were reported from the volatile oil of leaves of *C. tamala* by other workers earlier.[10–12] Composition of volatile oil of *C. malabatrum* was found to be distinct, but for the common occurrence of linalool and eugenol in both. GC-MS of volatile oil from the leaves of *C. malabatrum* revealed 11 constituents. Other than germacrene and ledene, all other constituents were reported from aerial parts of *C. malabatrum* recently.[13] Exhaustive chemical and pharmacological evaluation might be conclusive to designate leaves *C. malabatrum* as a substitute or as an adulterant for *C. tamala*.

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Pharmacognostic evaluation and phytochemical studies on leaves of *Vitex leucoxylon* Linn

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ABSTRACT

Introduction: *Vitex Leucoxylon* Linn. (family Verbenreae) is found commonly in India. This plant is a large deciduous tree, commonly known as Songarbhi (Marathi). It is small to large tree with a thick trunk and a spreading crown found almost throughout the Indian Deccan peninsular. The trees are generally found on river banks, streams and ponds. Recent pharmacological findings indicate that the crude alcoholic extract of its leaves possess anti-psychotic, anti-depressant, analgesic, anti-parkinsonian, anti-microbial activities, anti-inflammatory and wound-healing properties. However, no conclusive pharmacognostic study of its leaves has been performed yet. **Methods:** The present investigation deals with the qualitative and quantitative microscopic evaluation of the leaf material and the establishment of quality parameters including physicochemical and phytochemical evaluation. **Results:** Chief macroscopic and microscopic characters include midrib, lamina, venation pattern, stomata, epidermal trichomes, sclereids, powder microscopy and phytochemical evaluation done by standard methods. **Conclusion:** The results would serve as a useful gauge of standardization of leaf material and ensuring quality formulations.

Keywords: *Vitex leucoxylon,* microscopical characters, phytochemical ccreening.

INTRODUCTION

The World Health Organization (WHO) proves medicinal plants are the best source to obtain a variety of newer herbal drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand the properties, safety and efficacy.[1]

Standardization of natural products is a complex task due to the heterogeneous composition, in the form of the whole plant or plant part extracts obtained thereof. To ensure reproducible quality of herbal products, proper identification of the starting material is essential. The first step towards ensuring the quality of the starting material is authentication. Thus, in recent years there has been a rapid increase in the standardisation of selected medicinal plants of potential therapeutic significance.[2,3] Despite modern techniques, identification of plant drugs by pharmacognostic studies is more reliable.

According to who, the macroscopic and microscopic description of a medicinal plant is the first step towards establishing the identity and degree of purity of such materials and should be carried out before any tests are undertaken.[4] *Vitex Leucoxylon* Linn (Verbenreae) is a large deciduous tree, commonly known as Songarbhi (Marathi), an excellent herbal crude drug found in nature which has the composition of the entire essential constituents required for the normal and good health of human. It is a small to large tree with a thick trunk and spreading crown and is found throughout the Indian Deccan peninsular upto an altitude of 900 meters; and extends northwards up to Jhansi and parts of Bihar. The trees are generally found on river banks, streams and ponds. The roots and bark have astringent properties and the roots are also used as a febrifuge. The leaves are smoked for relieving headache and catarrh and are also used for medicinal baths in fever and anemia.[5]

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General pharmacological studies revealed anti-psychotic, anti-depressant, analgesic, anti-inflammatory, anti-parkinsonian and anti-microbial activities of the aqueous and ethanolic extracts of leaves of *Vitex Leucoxylon*[6] and also studied the anti-inflammatory and wound healing properties of the crude alcoholic extract of the leaves in acute inflammation model.[7] β-sitosterol, dimethyl terphthalate, vitexin, isovitexin, agnuside and aucubin were isolated from the leaves or barks of *Vitex Leucoxylon*.[8]

The main objective of present study was to perform pharmacognostic investigation and preliminary phytochemical screening of the leaves of *Vitex Leucoxylon* Linn.

MATERIALS AND METHODS

Collection and authentication

The leaves of *Vitex Leucoxylon* Linn were collected in the foothills of Yercaud, Salem, Tamil Nadu, in March 2011. The plant was identified and authenticated by the Botanical Survey of India, Southern Regional Centre, Coimbatore, Tamil Nadu, India. A voucher specimen [No.BSI/SRC/5/23/2011–12/Tech] has been kept in our museum for further reference. The leaves were separated and shade dried at room temperature for 10 days and coarsely powdered with a hand-grinding mill and the powder was passed through sieve no. s60.

Prepartion of the extract

The powdered material of *Vitex Leucoxylon* was extracted separately using the Soxhlet apparatus with different solvents[9] and an aqueous solvent for cold maceration. After extraction, the extracts were concentrated under reduced pressure.

Instruments used

Photographs of different magnifications were taken with Nikon Labphot 2 Microscopic Unit. For normal observations, a bright field was used. For the study of crystals, starch grains and lignified cells;polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background.[10]

MATERIALS AND METHODS

Collection of specimens

The plant specimens were collected from the foothill of Yercaud, Salem, Tamil Nadu, India. Care was taken to select healthy plants with normal organs. The required leaf samples were cut and removed from the plant and fixed in FAA (Formalin-5 ml + acetic acid-5 ml + 70% Ethyl alcohol-90 ml). After 24 hrs of fixing, the specimens were dehydrated with graded series of tertiary–butyl alcohol as per the schedule given by Sass (1940).[11] The specimens were castled into paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10–12 μm. De-waxing of the sections was done by customary procedure. The sections were stained with Toluidine blue as per the method published by O'Brien et al.[12] For studying the venation pattern and trichome distribution, paradermal sections (sections taken parallel to the surface of leaf) of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jefferey`s maceration fluid was prepared. Glycerin-mounted temporary preparations were made for macerated/cleared materials. Powdered materials of different parts were cleared with NaOH and mounted in glycerin medium after staining. Different cell component were studied and measured.

Photomicrographs

Microscopic descriptions of tissues were supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon Labhot 2 Microscopic unit. For the study of crystals, starch grains and lignified cells, polarized light were employed. Under polarized light they appeared bright against dark background. Magnifications of the figures were indicated by the scale–bars. Descriptive terms of the anatomical features are as given in the standard anatomy books.

Determination of physicochemical parameters

The dried powdered leaves were subjected to physicochemical analysis including fluorescence analysis.[13] moisture content, total ash, water soluble ash, acid insoluble ash, sulphated ash, alcohol soluble extractive and water soluble extractive[14] to determine the quality and purity of the plant materials.

Preliminary phytochemical screening

The dried powdered leaves were extracted with petroleum ether (60–80°C), chloroform and alcohol using Soxhlet apparatus and aqueous extraction by cold maceration. The solvents were completely removed and reduced pressure by using vacuum evaporator. All the extracts were

screened qualitatively for the presence of various groups of phytoconstituents using different chemical tests.[15]

RESULTS AND DISCUSSION

Pharmacognostic study

The *Vitex leucoxylon* Linn. plant is a large deciduous tree upto 15 m tall with 3–5 foliolate leaves and young branchlets are quadrangular, minutely pubescent and lenticellate. An attempt was made to study the pharmacognostical character of the leaves of *Vitex leucoxylon* Linn.

Macroscopical evaluation

Leaves compound, digitate or rarely trifoliate, opposite, decussate; rachis pulvinate, planoconvex in cross-section, minutely pubescent; petiolule 0.5–1.5 cm long, canaliculate, glabrous; leaflets 5 (rarely 3), lamina 7–11.5 × 2–3.5 cm, elliptic, apex acute to obtuse, base cuneate-attenuate, margin entire, chartaceous or thinly coriaceous, glaucous beneath, glabrous; midrib canaliculate above; secondary_nerves 6–14 pairs; tertiary_nerves reticulo-percurrent, not prominent. Inflorescence axillary corymbose cymes, minutely pubescent; flowers zygomorphic, sessile; corolla white with purplish pubescent; anther lobes purple.

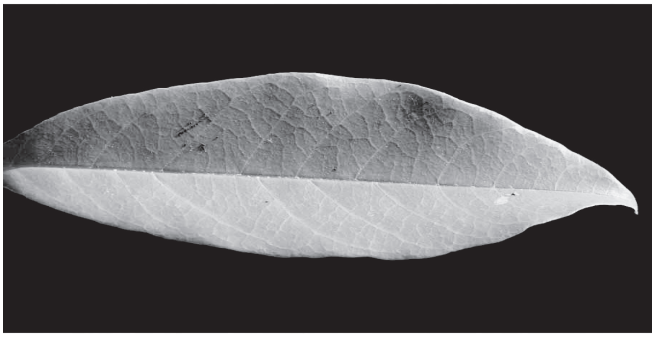

Figure No.?? Leaf of *Vitex leucoxylon* L.

MICROSCOPICAL STUDIES

Microscopic features of the leaflets

The leaflets exhibit dorsiventral symmetry with reference to the structure of the lamina and midrib (Fig. 1.1). The midrib is plano–convex; the adaxial side is flat and the abaxial side is semicircular. The midrib is 700 µm thick and 700 µm wide. The midrib consists of a thin epidermal layer of small thick-walled cells with a prominent cuticle. The ground tissue includes 5–9 layers of small, cirular compact parenchyma cells (Fig. 1.2).

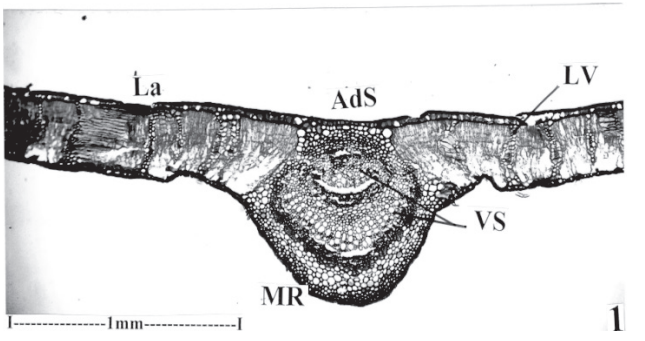

Figure 1.1. T.S. of leaflet through midrib.
(La-Lamina; AdS-Adaxial side; LV-Lateral Vein; VS-Vascular Strand; MR-midrib).

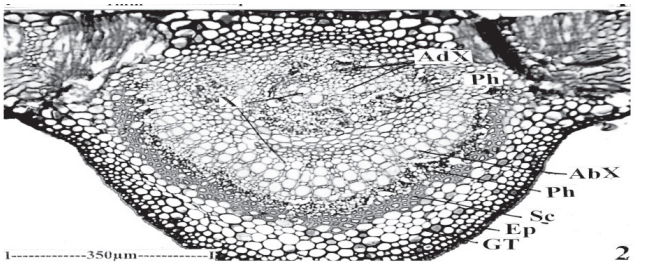

Figure 1.2. T.S. of midrib.
(AdX-Adaxial xylem; Ph-Phloem; AbX-Abaxial xylem; Sc-Sclerenchyma; Ep-Epidermis; GT-Ground tissue).

The vascular system of the midrib is somewhat complex. There is a wide, planoconvex sclerenchyma cylinder enclosing a wide prominent arc of abaxial vascular strand and adaxial small groups of irregularly disposed adaxial vascular strands. The abaxial arc consists of several parallel lines of 3–5 xylem elements situated along the abaxial part. Phloem occurs in uniformly thick arc abutting the xylem is on the upper part of the midrib, there is a narrow, thick band of vascular strands with a few xylem elements in short parallel rows and a wide and thick segment of phloem situated on the lower end of the xylem (Fig. 2.1). In addition to this adaxial segment, there are three or four small, less

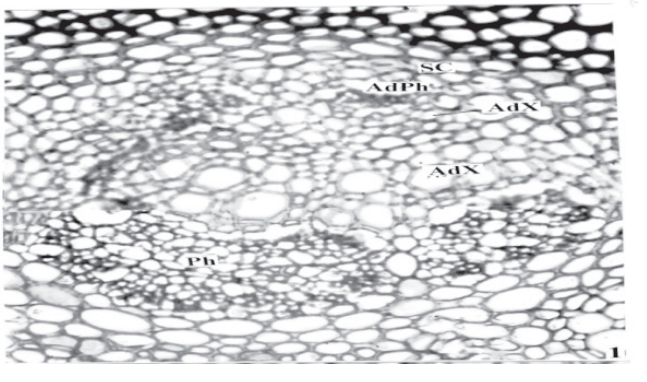

Figure 2.1. T.S. of midrib- adaxial vascular strands enlarged.
(Sc-Sclerenchyma; AdPh-Adaxial phloem; Adx-Adaxial xylem; Ph-Phloem).

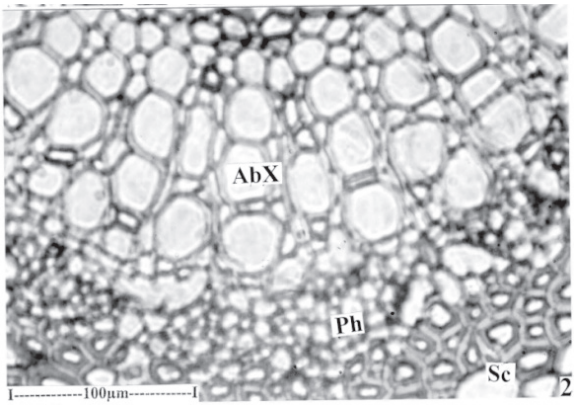

Figure 2.2. T.S. of midrib – abaxial vascular strands enlarged.
(Abx- Abaxial xylem; Ph-Phloem; Sc-Sclerenchyma).

prominent vascular strands with small clusters of xylem and phloem, xylem being placed at the abaxial end (Fig. 2.2). Thus, these are three groups of vascular strands, one abaxial wide, deeply bowl shaped, second adaxial thick flat plate and the third one being small less prominent, three or four nests.

Lamina

The lamina has smooth and even surfaces. It is 330 µm thick. It exhibits xeromorphic structure. The adaxial epidermal cells are vertically elongated, thick walled and heavily cuticularized; they are 20 µm thick. The abaxial epidermis is thin; the cells are narrowly rectangular and thick walled, measuring 10 µm thick (Fig. 3.1). The palisade mesophyll is 2 layered; the cells are narrow and vertically elongated; the palisade zone is 50 µm in height. The spongy mesophyll consists of 8–10 layers of small, lobed, loosely arranged parenchyma cells with wide intercellular spaces (Fig. 3.2).

The vascular strands of the lateral veins occur in vertical pillars at regular intervals. They have small collateral vascular bundles of xylem and phloem surrounded a layer of bundle sheath fibres with adaxial and abaxial extensions (Fig. 3.3).

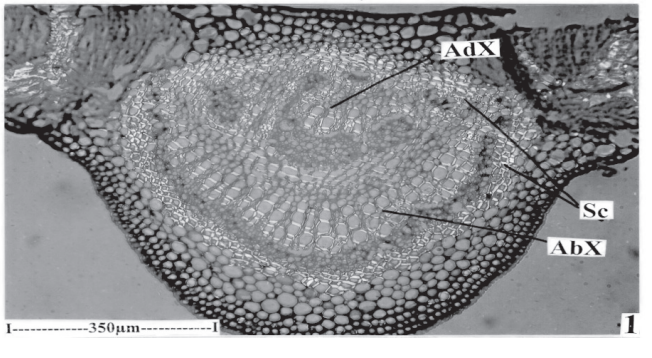

Figure 3.1. T.S. of midrib as seen under polarized light to show the lignified cells.
(AdX-Adaxial xylem; Sc-Sclerenchyma; AbX-Abaxial xylem).

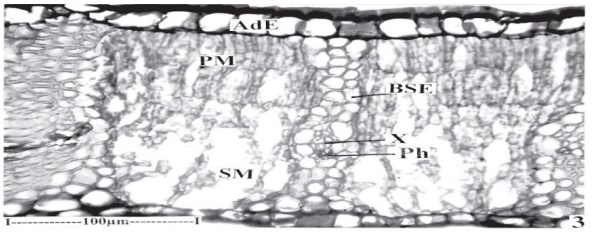

Figure 3.2. T.S. of lamina.
(PM-Palisade mesophyll; AdE-Adaxial Epidermis; LV-Lateral vein; SM-Spongy mesophyll).

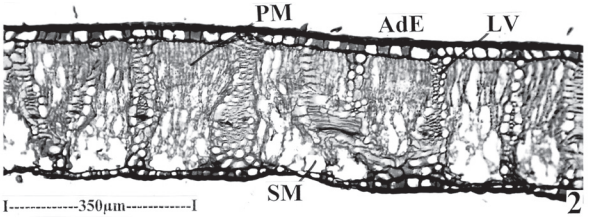

Figure 3.3. Vascular strand of the lamina enlarged.
(AdE-Adaxial epidermis; PM-Palisade mesophyll; BSE-Bundle sheath epidermis; SM-Spongy mesophyll).

Venation pattern

Venation was studied by paradermal sectioning (Fig. 4.1.). The venation is densely reticulate. Both major and minor veins are thick and straight with prominent bundle sheath cells (Fig. 4.2). The vein islets narrow, polygonal in outline and dense. The vein terminations are absent in most of the islets. When present, the terminations are short, thick and stumpy.

Stomata

Stomata are crowded in shallow depression and within the boundary of the vein–islets (areoles) the stomata are

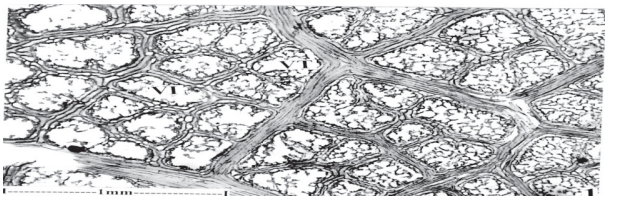

Figure 4.1. Paradermal sectional view of the venation pattern of the lamina.
(VT-Vein termination; VI-Vein islet).

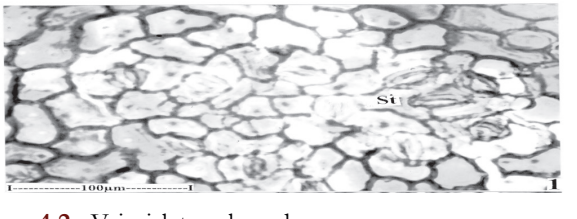

Figure 4.2. Vein-islets enlarged.
(St-Stomata).

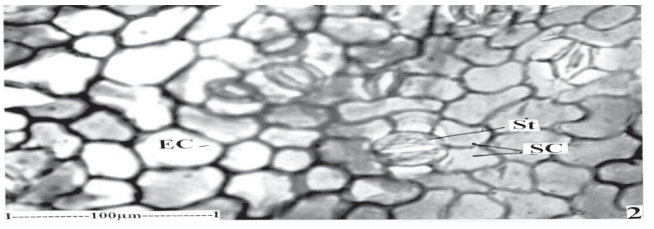

Figure 5.1. Stomata showing cyclocytic subsidery cells.
(EC-Epidermal cells; SC-Subsidery cells).

mostly cyclocytic type; they are surrounded by a circle of 4–6 subsidiary cells (Fig. 5.1). The guard cells are broadly elliptical, slightly thick walled, with wide stomatal pore. The stoma is 30 µm in length and breadth. The epidermal cells are narrow, thick walled with smooth walls.

Powder microscopy

Powdered preparation of the leaf exhibits the following elements when examined under the microscope:

Fragments of lamina

Small broken pieces of leaf–blades are frequently seen in the powder. They exhibit the venation. The venation is is closely reticulate with small and dense areoles (vein islets). They are variable in outline and very thick short and straight. There are primary areas with borders of thicker veins. Within such primary areas are the vein-islets with comparatively their veinlets. The vein terminations are not usually seen in most of the islets. Occasionally short, thick and simple (unbranched) terminations are seen in some of the islets (Figs. 6.1 and 6.2).

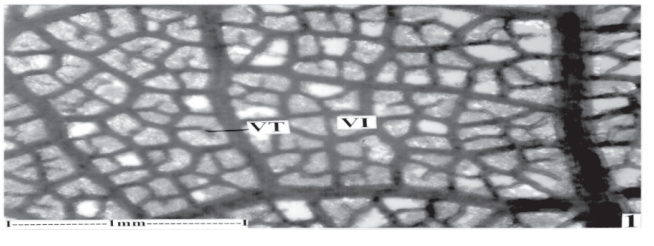

Figure 6.1. Leaf cleaved to show the venation pattern.
(VI-Vein islets; VT-Vein termination).

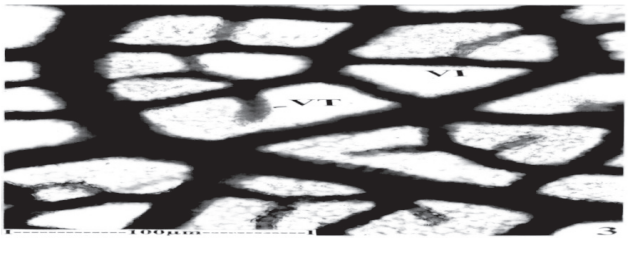

Figure 6.2. Venation pattern enlarged.
(VI-Vein islets; VT-Vein termination).

Epidermal peelings

Epidermal layers are often seen in the powder, both adaxial epidermal peeling and abaxial peelings are seen. The adaxial epidermal layer, as seen in surface view (Fig. 7.1) exhibits fairly thick anticlinal walls which are highly wavy. The cells appear amoeboid in outline (Fig. 9.2). The epidermis is apostomatic, having no stomata. The abaxial epidermal peelings are also seen in the powder. The abaxial layer is stomatiferous (having stomata). The stomata are seen in shallow areole and are highly crowded. They are cyclocytic type with circle of upto 6 subsidiary cells enclosing the guard cells.

Epidermal trichomes

Nonglandular epidermal trichomes are fairly common in the powder. They are 2 or 3 celled, unseriate, unbranched, and pointed at the tip. The walls are thick and the lumen is wide. The trichomes are 220–300 µm long and 20 µm thick (Fig. 7.2).

Sclereids

Sclereids of different shape and size are seen in the powder. They are broad, short elongated, fibre like cells. They may straight or curved. The walls are thick and the lumen is wide (Fig. 7.3) some of them have cell inclusions. The sclereids are 200–300 µm long and 20 µm wide.

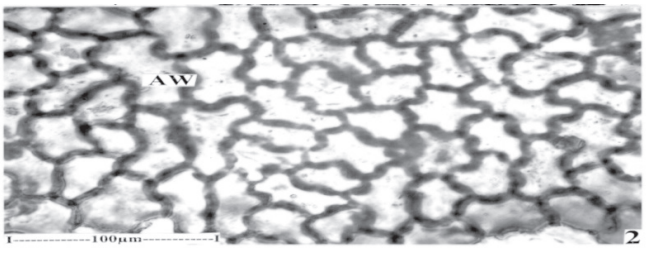

Figure 7.1. Adaxial epidermal cells-enlarged.
(AW-Anticlinal walls).

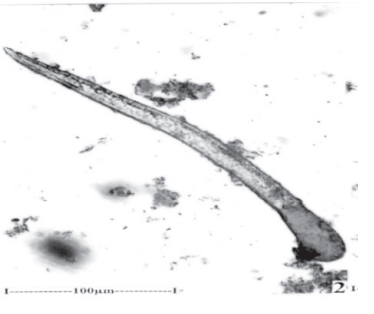

Figure 7.2. Epidermal trichomes.
(CL-Cell lumen; Scl-Sclereids)

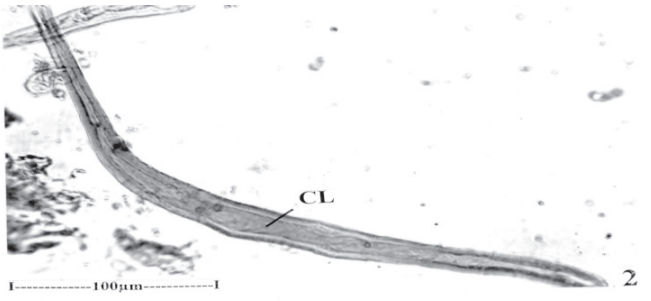

Figure 7.3. One sclereid enlarged.
(CL-Cell lumen; Scl-Sclereids).

Table 1. Physicochemical evaluation.

Parameter	% w/w
Moisture content	10.34 – 12.8
Foreign matters	0.5 – 0.7
Ash values	
Total ash	11.68 – 13.84
Acid insoluble ash	3.64 – 5.32
Water soluble ash	4.58 – 6.52
Sulphated ash	10.26 – 12.65
Extractive values	
Petroleum ether	2.53 – 5.22
Chloroform	2.51 – 4.98
Ethanol	14.97 –16.26
Aqueous	23.98 – 25.64

Physicochemical parameters

Fresh leaves of *Vitex leucoxylon* were collected and subjected to various physicochemical parameters such as moisture content, foreign matters, total ash, acid insoluble ash, water soluble ash, sulphated ash and the various extractive values are shown in Table 1.

Preliminary phytochemical studies

Qualitative chemical investigation of all the extracts of the selected plant was carried out to check the presence of several of phytoconstituents. It revealed the presence of alkaloids, carbohydrates, tannins, glycosides, flavanoids, saponins, protein and amino acids, steroids and gums and mucilage, etc (Table 2).

CONCLUSION

From the above discussion, it was concluded that the results would serve as a useful gauge standardization of the leaf material and ensuring quality formulations. The presence of phenol, tannin, carbohydrate, flavonoid and glycosides shows that this plant is potent neutraceutical agent. There is an urgent need for the documentation

Table 2. Preliminary phytochemical screening of leaves of *Vitex leucoxylon* Linn.

Plant Constituents	Petrolum ether Extract	Chloroform Extract	Alcoholic extract	Aqueous Extract
Alkaloids	–	–	+	+
Carbohydrates	–	–	+	+
Glycosides	–	–	+	+
Tannins & Phenolic compounds	–	–	+	+
Protein and amino acids	–	–	+	+
Flavonoids	–	+	+	+
Saponins	–	–	–	–
Steroids	–	–	+	–
Fixed oils and Fats	–	–	–	–
Gums and mucilage	–	–	–	+

Table 3. Reactions of powdered drug with different reagents.

Treatment	Colour
Powder as such	Pale green
Powder + conc. H_2SO_4	Yellowish black
Powder + conc. HNO_3	Yellowish brown
Powder + conc. HCL	Brown
Powder + 5% Iodine	Brownish yellow
Powder + 5M NaOH	Greenish brown
Powder + glacial acetic acid	Yellowish brown
Powder + 80% H_2SO_4	Yellowish brown

Table 4. Fluorescence Analysis.

Reagents	Day Light	UV Light
Drug powder	Pale green	Pale green
Drug Powder + 1M NaH	Greenish yellow	Green
Drug Powder + alcoholic 1M NaH	Dark green	Pale green
Drug powder + 1M HCl	Light brown	Faint green
Drug powder + 50% HNO_3	Light yellow	Pale green
Drug powder + 5% $Fecls_3$	Dark green	Greenish yellow
Drug powder + 80% H_2SO_4	Yellowish brown	Yellowish brown
Drug powder + water	Greenish yellow	Dark green
Drug powder + conc. H_2SO_4	Black	Greenish brown

of herbal drugs, systematic phytochemical and pharmacognostical studies of medicinal plants and their natural products.

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Phytopharmacopoeial specifications of *Garcinia indica* fruit rinds

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ABSTRACT

Introduction: Nature always stands as a golden mark to exemplify the outstanding phenomenon of symbiosis. Herbal medicines are the products of therapeutic experiences, of generations of practising physicians of indigenous systems of medicine for over hundreds of years. *Garcinia indica* Choisy Syn *Brindonia indica*, commonly known as kokum and belonging to Guttiferae family, is a popular plant native to certain regions of India. The plant has long been used in traditional Ayurvedic medicine for its magical effects in curing various diseases. **Objective:** To present a systematic investigation of phytopharmacopoeial standards for the fruit rinds of *Garcinia indica* by performing pharmacognostical parameters. **Material and Methods:** Fresh fruit rinds sample and dried power of the fruit rinds were studied macromorphologically and cytomorphologically along with its detail physicochemical and phytochemical investigation. Other WHO recommended parameters for standardizations were also investigated. **Results:** Macroscopic studies showed that fruit rind shape - round, oblong or oval with pointed tips and, were crowned by the four parted stalkless stigma with reddish black colour, aromatic odour and sour taste. The detailed microscopy revealed presence of parts of pericarp, oleo resin cells etc. the other physicochemical parameters were stands within the standardized range. **Conclusion:** The standardization parameters provide referential information for correct identification of the plant material and will also be useful in preparation of monographs on these plants.

Keywords: *Garcinia indica,* phytochemical characterization, phytopharmacopoeial standards, standardization.

INTRODUCTION

Medicinal herbs are moving from fringe to main stream use with a greater number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals. Recently, considerable attention has been paid to utilize eco-friendly and bio-friendly plant-based products for the prevention and cure of different human diseases. The Indian flora offers a variety of plants having medicinal properties. These plants can be exploited to find out effective alternative to synthetic drugs.[1] However, a key obstacle, which hindered the acceptance of the alternative medicines, is the lack of documentation and stringent control. Therefore, there is a need for documentation and stringent quality control of research work carried out on traditional medicines. With this backdrop it becomes extremely important to make an effort towards standardization of the plant material to be used as medicine. The process of standardization can be achieved by stepwise pharmacognostic studies. Correct identification and

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quality assurance of the starting materials is an essential prerequisite to ensure reproducible quality of herbal medicine, which will contribute to its safety and efficacy. Simple pharmacognostic techniques used in standardization of plant material include its morphological, anatomical and biochemical characteristics.[2] Herbal drugs or medicinal plants, their extracts and their isolated compound(s) have demonstrated spectrum of biological activities. These have been used and continued to be used as medicine in folklore or food supplement for various disorders. One such plant, *Garcinia indica* Choisy Syn *Brindonia indica*, commonly known as kokum and belonging to Guttiferae family, is a popular plant native to certain regions of India. The plant has since long been used in traditional Ayurvedic medicine for its magical effects to cure various diseases. In the traditional Indian system of medicine like Ayurveda and in various folk systems of medicine, the fruit rinds, seeds and leaves are used to treat various inflammatory ailments, rheumatic pain and bowel complaints. Chemically, it contains tannin, organic acids like (−)-hydroxycitric acid, hydroxycitric acid, lactone and citric acid; the anthocyanins, cyanidin-3-glucoside and cyanidin-3-sambubioside; and the polyisoprenylated phenolics, garcinol and isogarcinol. Preclinical studies have shown that kokum and/or some of its phytochemicals possess antibacterial, antifungal, anti-ulcerogenic, cardioprotective, anticancer, chemopreventive, free radical scavenging, antioxidant and anti-obesity effects.[3] But the pharmacognostical and phytochemical standardization of the fruit rinds were hitherto not reported. In view of the importance of this plant, the systematic investigation of phytopharmacopoeial standards for the species has been undertaken. The present study deals with pharmacopial standardization parameters for the fruit rinds of *Garcinia indica*. Emphasis is being laid on the areas of the most recent interest and those which have not been presented in earlier reports. The information will be used for further identification and preparation of plant monograph and will assist in standardization for quality, purity and sample identification.

MATERIALS AND METHODS

Collection of Samples

The fruit rinds of *Garcinia indica* was collected from the local area in Goa. Their identity and authentication was done by Department of Pharmacognosy, Marathwada Mitra Mandal's College of Pharmacy, Pune, by correlating their macromorphological characters with those given in literatures. The remaining fruit rind samples were dried in shade. Coarse powder (60 #) of dried fruit rinds of plants was stored for the microscopical study and phytochemical investigations.

Macromorphology

The entire fruit rinds of *Garcinia indica* and the powder was evaluated for their sensory profile by observing their colour, odour and taste along with some extra macroscopical characters as per standard WHO guidelines.[4–6]

Cytomorphology

The transverse sections of the fruit rinds were taken, cleared with clearing agent and mounted in glycerine water. Microscopy of dried fruit rind powder was studied for evaluating various parts present in the given fruit rind powder. The detail cytomorphological characters were observed under digital microscope (MOTIC-B1) and organ detection was reported according to the prescribed method.[7,8]

Microchemical Testing

For detection of cell wall composition and cell contents, the transverse sections of fruit rinds and powders were treated with different but specific staining reagents and observed under digital microscope (MOTIC-B1). The cell wall composition, cell contents and tissue detection was reported separately.[7,8]

Physicochemical Evaluation

Evaluation of crude drug ensures the identity of a drug and determines the quality and purity of drugs. The main reason behind the need forthe evaluation of crude drugs is biochemical variation in the drug, effect of treatment and storage of drugs and adulteration and substitutions.[9] Phytopharmacopoeial specification for the plant materials should be developed to enable the quality control chemists to verify and approve the materials.[10] The various physicochemical parameters were viz. ash values, extractive values and loss on drying. Determinations of these physicochemical constants were done as per the procedures mentioned in accordance with the WHO guidelines.[11,12]

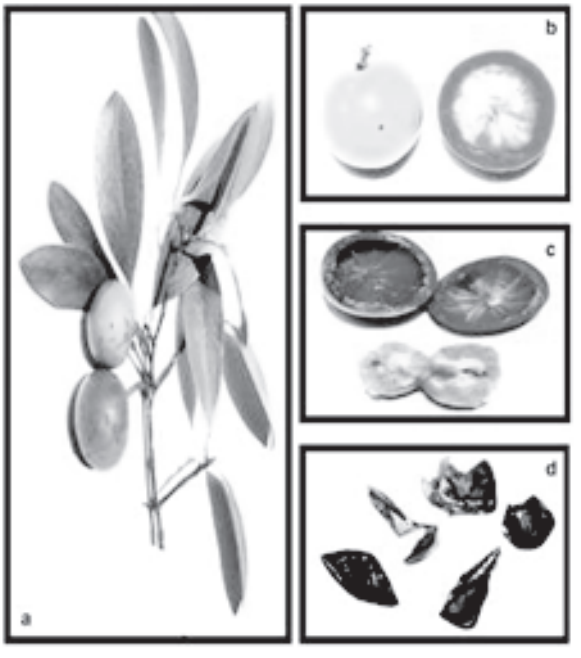
Preliminary Phytochemical Screening

The chemical evaluation includes qualitative chemical tests which are used for identification of various phytoconstituents present in the powered crude drug.[13,14]

RESULTS

Macrmorphological Description

Kokum tree is dioecious and grows up to a height of 12 to 20 m. The fruits were round, oblong or oval with pointed tips and, were crowned by the four parted stalkless stigma. The morphological details of the plants and fruit rinds are observed in Figure 1. The organoleptic evaluation of the fruit rinds and fruit rind powder revealed that the fruit rinds were dark red and the powder was reddish black in colour, with aromatic odour and sour taste. The results of morphological characters are mentioned in Table 1.



a. Plant with leaves and ripe Fruits
b. Raw fruits and ripe fruit with the inner seed arranged like in orange
c. Raw fruit rind and seeds
d. Dried rinds

Figure 1. Macrmorphological description.

Table 1. Macrmorphologcal description.

Sr.No.	Characters	Observation
Organoleptic characters		
1.	Colour	Dark red
2.	Odour	Aromatic
3.	Taste	Sour
Quantitative macromorphology		
4.	Size	2.5 – 3.7 cm in diameter
Macroscopical features		
5.	Shape	Globose or Spherical
6.	Type	Simple (Dry fruit- Dehiscent)

Cytomorphological Description

The outline of transverse section showed normal shape. Figure 2 revealed the transverse section of the pericarp, which was divided into three layers. The outermost was compactly arranged quadrangular or polygonal layer of cells is commonly called epicarp. The middle loosely arrange reticulated thin walled cell layer, is called mesocarp. Beneath the mesocarp, narrow elongated cells were arranged commonly called endocarp. The oleoresineous cells were also observed in a distributed manner within the mesocarp region which was stained by Sudan Red-III while the other starch grains were also observed in discrete manner which were stained by the N/50 Iodine solution. Figure 3 revealed the presence of epicarp region which also contributes to the attachment of a fruit with the tree through the region called as dessipiment. Figure 4 contributes to the presence of the oleoresin cells which were distributed throughout the mesocarp region. In

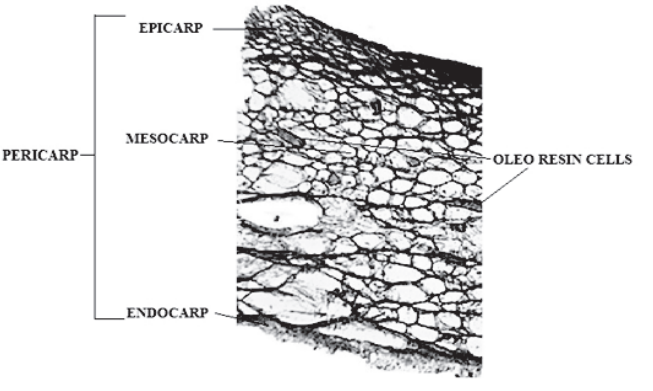


Figure 2. Cytomorphological description.

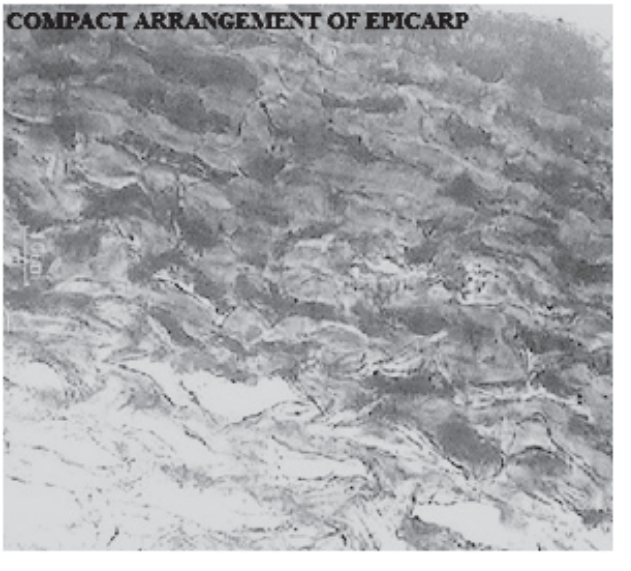


Figure 3. Compact arrangement of Epicarp.

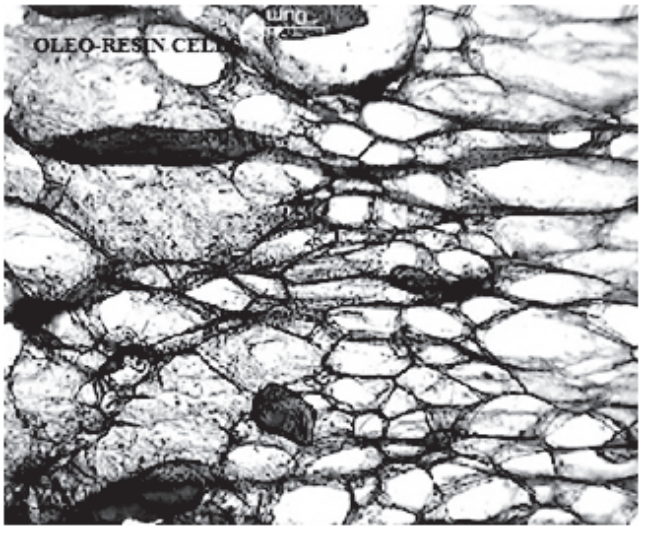


Figure 4. Oleoresin cells within Mesocarp.

powder microscopy Figure 5 showed the presence of fragments of epicarps which were compactly arranged cells. Figure 6 showed the presence of elongated lignified fibers which were stained by mixture of phloroglucinol solution and sulphuric acid in equal proportion the location of these fibers was found to be embedded within mesocarp region.

Microchemical Testing

Characterization of the cell wall components and cell contents were done with the help of microchemical testing

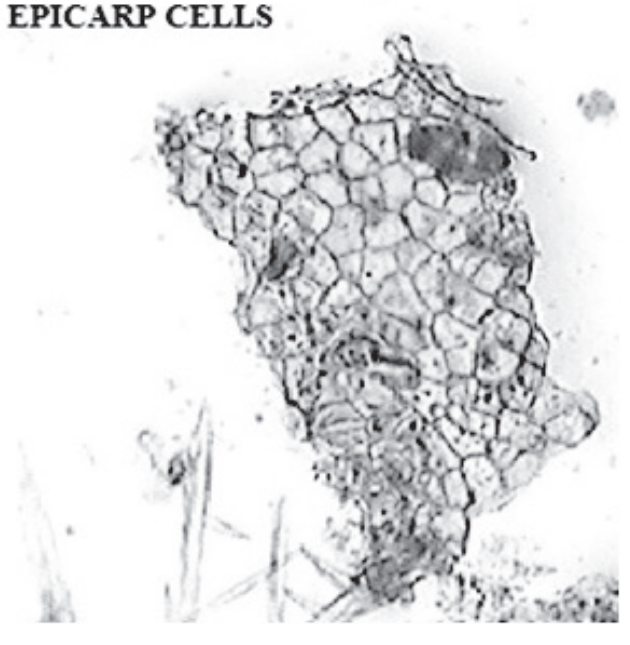


Figure 5. Powder Microscopy (Epicarp cells).

Figure 6. Powder Microscopy (Lignified fibers).

which revealed the presence of various types of cells and also characterization of these cells. It also signifies the tissues present in the fruit rind powder. The details of these microchemical testings are reported in Table 2.

Physicochemical Evaluation

Table 3 narrates the results of the physicochemical constants of powdered fruit rinds which lie within the limit; this signifies that the quality and purity of raw material was good enough. The moisture content of a drug will be responsible for decomposition of crude drugs either producing chemical change or microbial growth. So the moisture content of a drug should be determined and controlled. The result of the moisture

Table 2. Microchemical testing.

Sr. No.	Staining Reagents	Observations	Characteristics
1.	Phlorglucinol + conc. HCL	Pink	Lignified fibers
2.	Sudan red III	Red	Oil globules
3.	Iodine solution	Blue	Starch present

Table 3. Physico-chemical constants.

Sr. No.	Test	Result (%w/w)
1.	Moisture content	8.8
2.	Ash value:	
	Total ash	17.5
	Water Soluble ash	1.5
	Acid Insoluble ash	0.5
	Sulphated ash	14.3
3.	Extractive value:	
	Water soluble	60
	Alcohol soluble	75

content was found to be 8.80% which signifies that the drug was properly dried. The determination of ash is useful for detecting low grade products, exhausted drugs and excess of sandy or earthy matter. Different type of ash values are used for detection of crude drugs like, total ash, acid insoluble ash, water soluble ash and sulphated ash. The values were 17.5% for total ash, 1.5% for water soluble ash, 0.5% for acid insoluble ash and 14.3% for sulphated ash. The extracts obtained by exhausting crude drugs with different solvents are approximate measures of their chemical constituents. Extractive values of the crude drug were found to be 60% w/w for water soluble and 75% w/w for alcohol soluble extractive value.

Preliminary Phytochemical Screening

The powder drug was subjected to preliminary phytochemical screening for the presences of type of phytoconstituents. The powder was found to contain cardiac glycosides, saponin, flavonoids, tannins, citric acid and ascorbic acid. The results of the preliminary phytochemical screening are expressed in Table 4.

DISCUSSION

To ensure reproducible quality of herbal products, proper control of starting material is almost essential. Thus, in recent years there has been an emphasis on the standardization of medicinal plants of therapeutic potential. Despite the modern techniques, identification and evaluation of plant drugs by pharmacognostic studies is still more reliable, accurate and in expensive. According to World Health Organization (WHO) the macroscopic and microscopic description of a medicinal plant is the first step towards establishing the identity and purity and should be carried out before any tests are undertaken. Organoleptic evaluation is a technique of qualitative evaluation based on the study of morphological and sensory profiles of whole drugs. The organoleptic or macroscopic studies yielded important characteristics, such as the fractured surfaces of fresh and dried fruits, typical tongue sensitizing aromatic taste and aromatic and characteristic odour of fruits which are useful diagnostic characters. Microscopic evaluation is indispensable in the initial identification of herbs, as well as identifying small fragments of crude or powdered herbs, and in detection of adulterants as well as identifying the plant by characteristic tissue features. Every plant possesses a characteristic tissue structure, which can be demonstrated through study of tissue arrangement, cell walls, and configuration when properly mounted in stains, reagents and media.[9] From macromorphology and cytomorphology, the identification of *Garcinia indica* was confirmed which showed the special characters of Guttiferae family. The preparation of crude drug from the harvested drug plants involves cleaning or garbling to remove soil or other extraneous materials followed by drying which plays a very important role in the quality as well as purity of the material. The objectives of drying fresh material are, to aid in their preservation, to 'fix' their constituents, i.e., to check enzymatic or hydrolytic reaction that might alter the chemical composition of the drug, to facilitate subsequent comminution (grinding into a powder) and to ascertain their weight and bulk. Insufficient drying favours the spoilage by molds and bacteria and makes possible the enzymatic destruction of active principles. Not only is the ultimate dryness of the drug important, equally important is the rate at which the moisture is removed and the condition under which it is removed. If the rate is too slow, much spoilage may occur before the drying process is completed.[15] The limited moisture content of drug signifies that the drug was properly dried and the rate of drying was also good enough. The residue remaining after incineration of plant material is the ash content or ash value, which simply represents inorganic salts, naturally occurring in crude drug or adhering to it or deliberately added to it, as a form of adulteration. The total ash method is employed to measure the total amount of material remaining after ignition. This includes both 'physiological ash' which is derived from the plant tissue itself, and 'non-physiological ash,' which is the residue of the extraneous matter adhering to the plant surface. Acid-insoluble ash is a part of total ash and measures the amount of silica present, especially as sand, siliceous earth. Water-soluble ash is the water soluble portion of the total ash. These ash values are important quantitative standards. The ash content of the crude drug signifies that the sample of crude drug was of good quality without any adulterant or substitution. The percent extractives in different solvents indicate the quantity and nature of constituents in the extract. The colour of the extract sometimes may roughly indicate the physical and chemical features of constituents present and it was found that the crude drug contains more amount of polar constituents as compare to non

Table 4. Preliminary phytochemical screening.

Sr. No.	Parameters	Observations
1.	Cardiac glycosides	+
2.	Flavonoids	+
3.	Tannin	+
4.	Saponins	+
5.	Citric acid	+
6.	Ascorbic acid	+

polar with the highest proportion of colouring pigments. The plant material was subjected to preliminary photochemical screening by various chemical tests for qualitative detection of various chemical constituents where it indicates the presence of saponins, flavanoids and tannins and this data was useful for selection of solvent for extraction purpose.[16]

CONCLUSION

Establishing standards is an integral part of establishing the correct identity and quality of a crude drug. Before any drug can be included in the pharmacopoeia, these standards must be established. The majority of the information on the identity, purity and quality of the plant material can be obtained from its macroscopy, microscopy, physiochemical and phytochemical parameters. As there is no record on pharmacognostical work on fruit rinds of *Garcinia indica*, the present work was undertaken to produce some phytopharmacopoecial standards. Here the information collected was useful for further pharmacological and therapeutic evaluation of plant material.

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Antioxidant, analgesic and CNS depressant effects of *Synedrella nodiflora*

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ABSTRACT

Background: *Synedrella nodiflora* (L) Gaertn. belonging to the family Asteraceae contains steroids, reducing sugars, phenolic, flavonoids, saponins, tannins and triterpenoids. Therefore, this study is designed to investigate its antioxidant, analgesic and CNS depressant effects. **Materials and Methods:** The antioxidant activity of methanolic extract of *S. nodiflora* (SN) was determined using Folin Ciocalteu reagent, phosphomolybdenum method, free radical scavenging activity by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and the reducing power activity. Analgesic activity was done by acetic acid and formalin model and CNS depressant activity was measured by the hole cross and open field method. **Results:** The total phenols and total antioxidant capacity of SN was found to be 37.38 ± 2.01 mg/g equivalent of gallic acid and 433.37 ± 4.22 mg/gm equivalent of ascorbic acid. The percentage (%) scavenging of DPPH free radical was found to be concentration dependent and IC_{50} value was 125.89 ± 2.23 μg/ml while the IC_{50} value of standard ascorbic acid was found to be 16.76 ± 0.11 μg/ml. The reducing power of SN was found to be concentration dependent. Acetic acid and formalin models are peripherally acting analgesic methods. The oral administration of both doses (100 and 200 mg/kg b.wt.) of SN significantly ($p<0.001$) inhibited 40.06% and 61.83% writhing response induced by acetic acid whereas oral administration of the same doses of SN significantly ($p<0.001$) inhibited 56.96% and 62.60% itching response induced by formalin. The methanolic extract of SN, at the dose of 250 mg/kg and 500 mg/kg b.wt. produced significant ($P<0.001$) decrease of locomotion. This is the first report of CNS depressant activity of the plant. **Conclusion:** The extract showed moderate antioxidant, analgesic and CNS depressant potency. The present investigation suggests that SN may be a source of natural antioxidant with analgesic and CNS activity.

Keywords: *Synedrella nodiflora*, antioxidant, free radical, analgesic activity, CNS depressant.

INTRODUCTION

Free radicals cause depletion of immune system antioxidants, a change in gene expression and induce abnormal proteins and contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS.[1] It has been mentioned that the antioxidant activity of plants might be due to their phenolic, flavonoid, tannin and proanthocyanidin compounds.[2–3] Pain has been defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.[4] Pain can be constant (chronic) or fleeting and come and go (acute). There are several types of pain, namely 'nociceptive', 'neurogenic', 'neuropathic' and 'psychogenic', which are associated with a stimulation of nociceptors, damage to neuronal tissue, dysfunction of a nerve, or psychological factors, respectively.[5] The direct and indirect action of chemical mediators, such as arachidonic acid metabolites (prostaglandins and leukotrienes), peptides, serotonin, acetylcholine, cytokines, nitric oxide among others, which can be produced or released following

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tissue injury or by exogenous irritants (formalin, acetic acid), are responsible for the multiplicity of events that occur during pain transmission, in both the peripheral and central nervous systems.[6–8] Moreover, various free radicals as well as reactive oxygen species (ROS) are also responsible for the induction of short-term algesia[9] and trigger some second messengers, that are involved in sensitization of dorsal horn neurons and play a fundamentally important role in neuropathic pain.[10,11]

Synedrella nodiflora (L) Gaertn. belongs to the family *Asteraceae*. It is a small, annual weed, native to tropical America, found in the plains of India and in the Andamans. The methanol extract showed the presence of steroids, reducing sugars, phenolic compounds, saponins and tannins. Benzene and chloroform extracts showed the presence of steroids. Petroleum ether (40° – 60° C) extracts showed the presence of steroids and triterpenoids.[12]

The Asteraceae family consists of herbs which are known to accumulate substantial amount of flavonoids and to display anti-inflammatory, antioxidant, antimicrobial, analgesic and antipyretic properties.[13] In Ghana, *S. nodiflora* (L) Gaertn. weed is used for the treatment of epilepsy and pain.[14] In Malaysia and Indonesia, the plant is used for headaches, earaches, stomach aches and rheumatism.[15]

Literature reviews indicated that no studies combining the antioxidant, analgesic and CNS depressant of the leaves of *S. nodiflora* have so far been undertaken. Taking this in view and as a part of our ongoing research on Bangladeshi medicinal plants, the present study aimed to evaluate the antioxidant, analgesic and CNS activity of the methanolic leaves extract of *S. nodiflora.*

MATERIALS AND METHODS

Plant material

S. nodiflora plants were collected from Rajshahi in March 2009 and identified by Dr. M.A. Razzaque Shah, Tissue Culture Specialist, BRAC Plant Biotechnology Laboratory, Bangladesh. A voucher specimen for this collection has been maintained in the Bangladesh National Herbarium (Voucher Specimen No-34479), Dhaka, Bangladesh.

Preparation of the extract

The leaves of plant were first washed with water to remove adhering dirt and then dried at 45°C for 36 hrs in an electric oven, then powdered with a mechanical grinder, passed through sieve #40, and stored in a tight container. The dried powdered material (1 kg) was taken in a clean, flat bottomed glass container and soaked in methanol for seven days. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. The total filtrate was concentrated to dryness, *in vacuo* at 40°C to render the methanol extract (390 g) of brownish red color.

Drugs and chemicals

The active drugs Indomethacin and Diazepam were generous gift samples from Square Pharmaceuticals Ltd., Bangladesh. Acetic acid was obtained from Merck, Germany. Tween-80 was obtained from BDH Chemicals, UK. Formalin was purchased from CDH, India. Normal saline solution was purchased from Beximco Infusion Ltd., Bangladesh. All chemicals used were of analytical reagent grade.

Animals

Young Long-Evans rats of either sex weighing about 80–120 gm were used for the experiment. The rats were purchased from the Animal Research Branch of the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). They were kept in standard environmental conditions (at 24.0 ± 0°C & 55–65% relative humidity and 12 hour light/dark cycle) for one week for acclimation after their purchase and fed ICDDRB formulated rodent food and water *ad libitum*. The set of rules followed for animal experiment were approved by the institutional animal ethical committee.[16]

Acute toxicity

The 50% lethal dose (LD_{50}) of the SN in rats was estimated by the up and down method.[17] Doses were adjusted up or down by a constant multiplicative factor (1.5) depending on the previous outcome.

IN VITRO ANTIOXIDANT ACTIVITY

The amount of phenolic compounds

The total phenolic content of methanolic extract of SN was determined using Folin–Ciocalteu reagent.[18] The content of total phenolic content in the extract of SN was calculated from the regression equation of the calibration curve ($y = 0.0138x + 0.1275$, $r^2 = 0.988$) and expressed as Gallic acid equivalents (GAE).

Determination of total antioxidant capacity

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method.[19] The assay is based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. The total antioxidant in the extract of SN was calculated from the regression equation of the calibration curve ($y = 0.0043x + 0.1503$, $r^2 = 0.8874$) and expressed as Ascorbic acid equivalent (AAE).

Free radical scavenging activity measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH)

The free radical scavenging activity of the extract, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined by the method described by Braca.[20] The percentage inhibition activity was calculated from $[(A_0–A_1)/A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard. IC_{50} value was calculated from the equation of line obtained by plotting a graph of concentration (µg/ml) versus % inhibition.

Reducing power activity

The reducing power of SN was determined according to the method described by Oyaizu.[21] Increased absorbance of the reaction mixture indicated increased reducing power.

IN VIVO ANALGESIC ACTIVITY

Acetic acid-induced writhing test

The analgesic activity of the samples was also evaluated using acetic acid-induced writhing model in rats.[22] In this method, acetic acid is administered intraperitonially to the experimental animals to create pain sensation. The test samples (100 and 200 mg/kg body weight) and vehicle (1% Tween-80 in water) were administered orally 30 min before intraperitoneal administration of 0.7% v/v acetic acid but indomethacin (10 mg/kg) was administered orally 15 min before injection of acetic acid. After an interval of 5 min, the rats were observed for specific contraction of body referred to as 'writhing' for the next 10 min. Full writhing was not always accomplished by the animal, because sometimes the animals started to give writhing but they did not complete it. This incomplete writhing was considered as half-writhing. Accordingly, two half-writhing were taken as one full writhing. The number of writhes in each treated group was compared to that of a control group while Indomethacin (10 mg/kg) was used as a reference substance (positive control). The percent inhibition (% analgesic activity) was calculated by

$$\% \text{ inhibition} = \{(A - B)/A\} \times 100$$

Where, A = Average number of writhing of control per group; B = Average number of writhing of test per group.

Formalin test

The antinociceptive activity of the drugs was determined using the formalin test described by Sharma.[22] Control group received 5% formalin. 20 µl of 5% formalin was injected into the dorsal surface of the right hind paw 60 min after administration of SN (100 and 200 mg/kg, p.o.) and 30 min after administration of indomethacin (10 mg/kg, i.p.). The rats were observed for 30 min after the injection of formalin, and the amount of time spent licking the injected hind paw was recorded. The first 5 min post formalin injection is referred to as the early phase and the period between 15 and 30 min as the late phase. The total time spent licking or biting the injured paw (pain behavior) was measured with a stop watch. The percent inhibition (% licking activity) was calculated using the above equation.

CNS DEPRESSANT ACTIVITY

Hole cross test

The method was carried out as described by Takagi et al.[23] A steel partition was fixed in the middle of a cage having a size of 30 × 20 × 14 cm. A hole of 3 cm diameter was made at a height of 7.5 cm in the center of the cage. Twenty animals were divided into four groups with five rats in each group. Group I animals received vehicle (1% Tween-80 in water, 10 ml kg^{-1} p.o.), animals of Group II received diazepam at 1 mg kg^{-1} body weight (p.o.) while animals of Groups III and IV were treated with 250 and 500 mg kg^{-1} body weight (p.o.) of the SN. The number of passages of a rat through the hole from one chamber to other was counted for a period of 3 min on 0, 30, 60, 90 and 120 min after oral administration of test drugs

Open field test

The animals were treated as discussed above. The experiment was carried out according to the methods described by Gupta.[24] The floor of an open field of half square meter was divided into a series of squares each alternatively colored black and white. The apparatus

had 40 cm height wall. The number of squares visited by the animals were counted for 3 min, on 0, 30, 60, 90 and 120 min after oral administration of test drugs.

STATISTICAL ANALYSIS

All values were expressed as the mean ± SEM of three replicate experiments. The figure of DPPH and reducing power was done by using Graph Pad Prism (version 5.0; Graph Pad, San Diego, CA, USA). All *in vivo* data were analyzed by using student's t test compared with control and each value represents the mean ± SEM of 5 experiments. $*P<0.05$; $**P<0.001$ were considered to be statistically significant.

RESULTS

The qualitative chemical analysis of *S. nodiflora* showed the positive result for the presence of phenol and flavonoid.

ACUTE TOXICITY

Oral administration of graded doses of SN (500–5000 mg/kg, body weight) did not cause any death in the different dose groups. The LD_{50} value for oral administration of the plant extract was found to be greater than 5000 mg/kg.

IN VITRO ANTIOXIDANT ACTIVITY

Total phenolic contents

The total phenolic content was found to be 37.38 ± 2.01 mg/g plant extract (in GAE) in crude extract of *S. nodiflora*. The results are shown in Table 1.

The GAE and ASC values are expressed as means ± SEM of triplicate experiments

Total antioxidant capacity

Total antioxidant capacity of *S. nodiflora* was expressed as the number of equivalents of ascorbic acid (Table 1).

Table 1. Total amount of plant phenolic compounds and total antioxidant capacity of methanolic extract of *S. nodiflora*.

Sample	Total Phenols mgg^{-1} plant extract (in GAE)[a]	Total antioxidant capacity mg g^{-1} plant extract (in ASC)[b]
Synedrella (200 µg/ml)	37.38 ± 2.01	433.37 ± 4.22

[a]Gallic acid equivalents (GAE, mg/g of each extract) for the total phenolic content.
[b]Ascorbic acid equivalents (mg/g of each extract) for the total antioxidant capacity.

Total antioxidant capacity of SN was found to be 433.37 ± 4.22 mg/gm equivalent of ascorbic acid.

DPPH radical scavenging activity

The percentage (%) scavenging of DPPH free radical was found to be concentration dependent i.e. concentration of the extract between 25–200 µg/ml greatly increased the inhibitory activity (Figure 1) with the IC_{50} value of IC_{50} 125.89 ± 2.23 µg/ml, while IC_{50} value of standard ascorbic acid was found to be 16.76 ± 0.11 µg/ml.

Reducing power ability

For the measurement of the reductive ability, we investigated the Fe^{3+} to Fe^{2+} transformation in the presence of SN and compared with standard ascorbic acid as shown in Figure 2. The reducing power of SN was found to be concentration dependent.

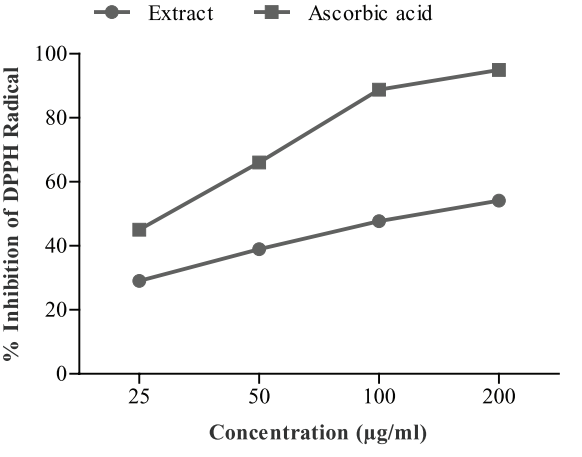

Figure 1. Free radical scavenging activity of different concentrations of methanolic extract of SN by DPPH radicals.

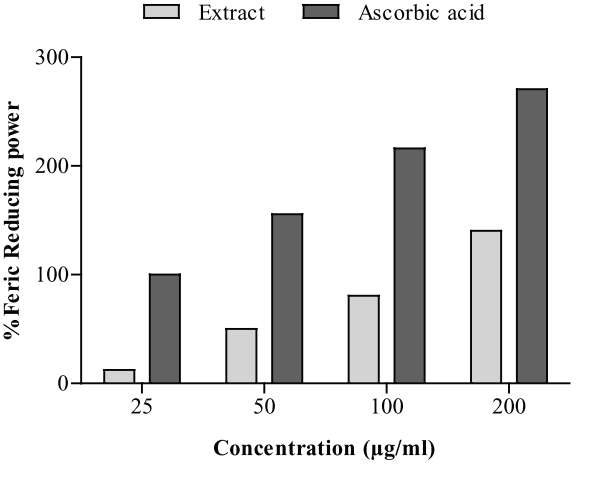

Figure 2. Reducing power of MeOH extract of SN and ascorbic acid by spectophotometric detection of Fe^{3+} to Fe^{2+} transformation.

IN VIVO ANALGESIC ACTIVITY

Acetic acid-induced writhing test

Table 2 shows the effects of the extract on acetic acid-induced writhing in rats. The oral administration of both doses of SN significantly ($p<0.001$) inhibited writhing response induced by acetic acid in a dose dependent manner.

Formalin test

SN (100 and 200 mg/kg, p.o.) significantly ($P<0.001$) suppressed the licking activity in either phase of the formalin-induced pain in rat in a dose dependant manner (Table 3). SN, at the dose of 200 mg/kg body weight, showed more licking activity against both phases of formalin-induced pain than that of the standard drug indomethacin.

CNS DEPRESSANT ACTIVITY

Hole cross test

The methanolic extract of SN, at the dose of 250 mg/kg and 500 mg/kg b.w. produced significant ($P<0.001$) decrease of locomotion from its initial value during the period of experiment (Table 4).

Open field test

Results of open field test are presented in Table 5 for the crude extract of SN. The number of squares traveled by the rats were suppressed significantly from the second observation period at both dose levels (250 mg/kg and 500 mg/kg b.w.) of SN. The results were dose dependent and statistically significant.

Table 2. Effect of SN on acetic acid induced writhing in rats.

Groups	Treatment	Dose, route	No. of writhing	% inhibition
Group-I	1% Tween 80 in water	0.1 ml/10 gm body weight, p.o	26.33 ± 0.55	
Group-II	Indomethacin	10 mg/kg, p.o	10.83 ± 1.22**	58.8
Group-III	SN	100 mg/kg, p.o	14.2 ± 1.69**	40.06
Group-IV	SN	200 mg/kg, p.o	10.05 ± 0.56**	61.83

Each value represents the mean ± SEM of 5 experiments. **$P<0.001$ considered statistically significant, using student's t test compared with control, SN represents methanolic extract of *S. nodiflora*

Table 3. Effect of SN in hind paw licking in the formalin test in rats.

Groups	Dose, route	Early phase (Sec)	% protection	Late phase (Sec)	% protection
Group-I (Distilled water)	10 ml/kg, p.o	35.67 ± 1.38	–	46.0 ± 1.03	–
Group-II (Indomethacin)	10 mg/kg, i.p	16.83 ± 0.90**	52.8	21.83 ± 0.70**	52.53
Group-III (SN)	100 mg/kg, p.o	28.2 ± 0.76*	20.94	19.8 ± 0.72**	56.96
Group-IV (SN)	200 mg/kg, p.o	15.1 ± 0.54**	57.66	17.2 ± 0.99**	62.60

Each value represents the mean ± SEM of 5 experiments. **$P<0.001$ considered statistically significant, using student's t test compared with control, SN represents methanolic extract of *S. nodiflora*

Table 4. Effect of SN on hole cross test in rats.

Group	Dose, Route	Number of movement				
		0 min	30 min	60 min	90 min	120 min
Group-I (1%tween80in water)	10 ml/kg, p.o.	118.4 ±1.20	118±1.30	115.4±0.50	117.4 ± 1.16	118 ± 0.70
Group-II (Diazepam)	1 mg/kg, p.o.	117.2 ± 1.15	±64.6 ±.43**	40.8 ±.58**	18.8 ± .86**	9.6 ± 0.50**
Group-III (SN)	250 mg/kg, p.o.	118.4 ± 0.81	±75.2 ±1.52**	50.6 ±1.11**	30.5 ± 0.05**	25.6 ± 0.71**
Group-IV (SN)	500 mg/kg, p.o.	118.0 ± 1.43	±62.8 ± .03**	40.2 ± .92**	22.6 ± 1.02**	15.4 ± 0.50**

Each value represents the mean ± SEM of 5 experiments. **$P<0.001$ considered statistically significant, using student's t test compared with control, SN represents methanolic extract of *S. nodiflora.*

Table 5. Effect of SN on open field test in rats.

Group	Dose, Route	Number of Movements				
		0 min	30 min	60 min	90 min	120 min
Group-I (1% tween 80 in water)	10 ml/kg, p.o.	12.8 ± 1.15	13± 1.41	13.6± 0.92	14.± 0.86	14± 0.54
Group-II (Diazepam)	1 mg/kg, p.o.	11.2± 0.58	6± 0.70**	4.0±0.83**	2.4±0.81**	1.8±0.37**
Group-III (SYNO)	250 mg/kg, p.o.	13± 0.70	7.2± 0.60*	5.0±0.55**	4.3±0.37**	2.8±0.37**
Group-IV (SYNO)	500 mg/kg, p.o.	12.2± 0.66	6.5± 0.37*	4.5±0.69**	2.8±0.37**	1.6±0.50**

Each value represents the mean ± SEM of 5 experiments. *P <0.05 & **P<0.001 considered statistically significant, using student's t test compared with control, SN represents methanolic extract of S. nodiflora.

DISCUSSION

To determine the efficacy of natural antioxidants either as pure compounds or as plant extracts, a great number of *in vitro* methods have been developed in which antioxidant compounds act by several mechanisms. The knowledge of total antioxidant activity can be useful in the analysis of changes in the plasma antioxidant activity related to oxidative stress, or the understanding of structure–activity relationships of pure antioxidant species. The phosphomolybdenum method was based on the reduction of Mo(VI) to Mo(V) by the compounds having antioxidant property and was successfully used to quantify vitamin E in seeds.[25] DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule[26] and is usually used as a substrate to evaluate the antioxidant activity of a compound.[27] Based on the data obtained from this study, DPPH radical scavenging activity of SN (IC_{50} 125.89 ± 2.23 μg/ml) was lower than the standard ascorbic acid (IC_{50} 16.76 ± 0.11 μg/ml). Moreover, it was revealed that SN did show the proton donating ability and could serve as a free radical inhibitor or scavenger. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.[28] Because a substance may act as an antioxidant due to its ability to reduce ROS by donating hydrogen atom,[29,30] the ferric reducing property of plant extracts (Fig. 2) implies that they are capable of donating hydrogen atom in a dose dependent manner. Polyphenolic compounds, like flavonoids and phenol, commonly found in plants have been reported to have multiple biological effects, including antioxidant activity.[31] Phenolic compounds are understood to induce the cellular antioxidant system; increase approximately 50% cellular glutathione concentration. Flavonoids are important in the modulation of γ-glutamylcysteine synthase in both cellular antioxidant defenses and detoxification of xenobiotics.[32] Flavonoid and phenol have been reported from SN.[12] We have also got phenol content 37.38 ± 2.01 mg/g and this may be the cause for the antioxidant activity in different models.

Acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics and represented pain sensation by triggering localized inflammatory response. Such pain stimulus led to the release of free arachidonic acid from the tissue phospholipid.[33] The response was thought to be mediated by peritoneal mast cells,[34] acid sensing ion channels[35] and the prostaglandin pathways.[36] The organic acid has also been postulated to act indirectly by inducing the release of endogenous mediators, which stimulates the nociceptive neurons that are sensitive to NSAIDs and narcotics.[37] It is well known that non-steroidal, anti-inflammatory and analgesic drugs mitigate the inflammatory pain by inhibiting the formation of pain mediators at the peripheral target sites where prostaglandins and bradykinin are proposed to play a significant role in the pain process.[38] In addition, it was suggested that non narcotic analgesics produce their action by interfering with the local reaction to peritoneal irritation thereby reducing the intensity of afferent nervous stimulation in the acetic acid induced writhing test, a model of visceral pain.[39] Therefore, it is likely that SN might have exerted its peripheral antinociceptive action by interfering with the local reaction caused by the irritant or by inhibiting the synthesis, release and/or antagonizing the action of pain mediators at the target sites and this response in agreement with the previous studies with other parts of *S. nodiflora*.[13] The above findings clearly demonstrated that peripheral mechanisms are involved in the antinociceptive action of SN.

On the other hand, the formalin model normally postulates the site and the mechanism of action of the analgesic.[40] This biphasic model is represented by neurogenic (0–5 min) and inflammatory pain (15–30 min), respectively.[41] The mechanism by which formalin triggers C-fibers activation remained unknown for a relatively long time. Recently, however,[42] it was demonstrated that formalin activates primary afferent neurons through a specific and direct on TRPA1, a member of the transient receptor potential family of cation channels, expressed by a subset of C-fiber nociceptors, and this effect is accompanied by increased influx of Ca^{2+} ions. TRPA1 cation channels at primary sensory terminals were also reported to mediate noxious mechanical stimuli.[43] These experiments suggest that Ca^{2+} mobilization through TRPA1 cation channels is concomitant with noxious chemicals and mechanical stimuli as they produce their analgesic action. It is likely that the inhibitory effect of SN to the pain response is due to inhibiting the increase of the intracellular Ca^{2+} through TRPA1, presumably evoked by formalin. So, the leaf extract of *S. nodiflora* may contain substances that affect the metabolism of Ca^{2+}. Literature survey revealed that tannins, triterpenoids and flavonoids are the major phytoconstituents of *S. nodiflora*.[12] Flavonoids, for example, have been found to suppress the intracellular Ca^{2+} ion elevation in a dose dependent manner, as well as the release of proinflammatory mediators such as TNFα.[44]

The effect of methanol extract of *S. nodiflora* on CNS was evaluated. The result indicated that the extract significantly decreased the locomotor activity (as shown by the results of the open field and hole cross tests). The locomotor activity is a measure of the level of excitability of the CNS[45] and any decrease of this activity may be closely related to sedation resulting from depression of the central nervous system.[46] Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system. Different anxiolytic, muscle relaxant, sedative-hypnotic drugs are elucidation their action through $GABA_A$, therefore it is possible that SN may acts by potentiating GABAergic inhibition in the CNS via membrane hyperpolarization which leads to a decrease in the firing rate of critical neurons in the brain or may be due to direct activation of GABA receptor by the extracts.[47] Many researches have shown that plant containing flavonoids, saponins and tannins are useful in many CNS disorders.[48] Earlier investigation on phytoconstituents and plants suggests that many flavonoids and neuroactive steroids were found to be ligands for the $GABA_A$ receptors in the central nervous system; which led to the assumption that they can act as benzodiazepine like molecules.[49] Previous phytochemical investigations also showed the presence of such types of phytoconstituents and they are responsible for CNS depressant activity. *But this is the first report demonstrating the* CNS depressant *activity of leaves of methanolic extract of S. nodiflora.*

CONCLUSION

In conclusion, this work has demonstrated that that the plant extracts from the leaf of *S. nodiflora* (L) Gaertn. (Asteraceae) possess moderate antioxidant, analgesic and CNS depressant potential, thereby lending support to the traditional use of the plant in painful and inflammatory disorders. However, further studies are needed to be conducted to understand the exact mechanisms of such actions and to isolate the active principles responsible for the observed activity.

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Evaluation of the anti diabetic activity of column fractions obtained from the bark extract of *Soymida febrifuga* A. Juss

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ABSTRACT

Diabetes has become a worldwide problem afflicting humans irrespective of age. Even though a number of synthetic drugs are available for the treatment of diabetes, plant drugs are generally preferred due to the assumption that they have less side effects and low cost. This study reports the hypoglycaemic and antihyperglycaemic actions of the methanolic bark extract of *Soymida febrifuga* A. Juss. (fam: Meliaceae) in euglycaemic and alloxan-induced diabetic rats, respectively. The results revealed that three column fractions obtained from the methanol extract of *S. febrifuga* possess maximum hypoglycaemic and antihyperglycaemic activities 6 h after treatment. At a dose of 200 mg/kg, the relatively nonpolar column fraction obtained by using 20% chloroform in acetone as an eluent was by far the most potent fraction which showed comparable activity with that of the standard drug glibenclamide (10 mg/kg). At a dose of 200 mg/kg, the same column eluate showed maximum antihyperglycaemic effect reducing blood glucose level by 33.00%. At a dose of 10 mg/kg, the reference drug glibenclamide brought about 33.46% reduction of blood glucose level. In light of the results obtained from the current study, it could be concluded that the bark of *S. febrifuga* has genuine antidiabetic activity.

Keywords *Soymida febrifuga*, Meliaceae, methanol extract, euglycaemic, hypoglycaemic effect, alloxan-induced antihyperglycaemic activity.

INTRODUCTION

Interest in herbal medicines is growing day-by-day because nature can cure many diseases. Diabetes has become a very common ailment afflicting humans irrespective of age. It is a worldwide problem, and India is not exceptional. Even though a number of synthetic drugs are available for the treatment of diabetes, plant drugs are generally preferred due to the assumption that they have less side effects and low cost.

Soymida febrifuga A. Juss. (fam: Meliaceae) commonly called Indian Red Wood is an endemic plant that grows wild in all dry deciduous forests of Andhra Pradesh, India.[1] The plant is easily identified by its grayish green paripinnate alternate leaves, and the red petioles and veins that the young leaves have. Traditionally, it is used for the treatment of diseases like rheumatoid arthritis, asthma and vaginal infections.[2] It is often claimed that the extracts of the plant have good activity against ulcers, tridosha fevers, leprosy, dysentery and diarrhea.[3] It has also been reported that the extracts of the plant have antimalarial activity similar to that of the cinchona bark. Decoction of the crushed bark is used for tongue sores, fixing loose teeth, gum infection and cough.[4]

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Simonsen et al.[5] showed that the bark extract of *S. febrifuga* possess significant *in vitro* antiplasmodial activity against *Plasmodium falciparum*. The extract also showed a dose dependent anti-inflammatory activity in Carrageenan-induced rat paw oedema, which was comparable to NSAIDs like naproxen, ibuprofen, and piroxicam.[6] Methyl angolensate, a natural tetranortriterpenoid isolated from *S. febrifuga* root calluses has been reported to have anticancer activity against T-cell leukemia, and chronic myelogenous leukemia.[7]

Several chemical constituents including quercetin, myricetin, quercetin 3-*O*-L-rhamnoside, lupeol, luteolin and tetranortriterpenoids have been isolated from the different parts of *S. febrifuga*.[8,9] Some of these constituents have been shown to possess antioxidant activity.[10]

In view of the wide medicinal uses of the various parts of *S. febrifuga*, it was deemed prudent to assess the effect of the bark extract of the plant on blood glucose levels in euglycaemic as well as in alloxan-induced diabetic rats.

MATERIALS AND METHODS

Materials

Plant materials

The bark of *S. febrifuga* was collected from the forest areas of Rajahmundry, East Godavari District, Andhra Pradesh, India. Immediately after collection the plant materials were authenticated by Dr. VS Raju, Department of Botany, Kakatiya University, and voucher specimens were deposited at KVSR Siddhartha College of Pharmaceutical Sciences, Vijayawada, for future reference.

Chemicals and drugs

Glibenclamide was a generous gift from Dr. Reddy's Foundation, Hyderabad, India. Alloxan monohydrate was purchased from Sigma-Aldrich, Germany. Assay kits (GOD-POD) were purchased from Beacon Diagnostics Ltd., Navasari, India. All other chemicals and solvents used were of analytical grade procured from local suppliers.

Animals

Wistar albino rats weighing 180–200 g were purchased from Mahaveer agencies, Hyderabad, India, and used for the studies after obtaining permission from institutional animal ethical committee (CPCSEA Reg. No. 146/1999). Experimental animals were housed in standard polypropylene cages, maintained under standard laboratory conditions (12 hour light/dark cycle; at an ambient temperature of 25 ± 5ºC; 35%–60% of relative humidity) and fed with standard rat pellet diet and water *ad libitum*.

Methods

Extraction and phytochemical screening

Barks of *S. febrifuga* were dried in shade and powdered in a mechanical grinder. Chloroform, methanol and aqueous extracts were prepared by maceration with sufficient amounts of the respective solvents for 7 days with intermittent stirring. The contents of the flask were then filtered and the mark further macerated with fresh solvents for an additional 3 days. After filtration, the combined extracts were concentrated to dryness under reduced pressure, first kept in desiccators, weighed and then stored in a refrigerator for future use. Preliminary phytochemical screening was carried out on the methanol bark extract of *S. febrifuga* following standard procedures.[11,12,13] The extract was then subjected to column chromatography using hexane, benzene, chloroform, acetone and methanol. Among the collected fractions only three fractions, namely, the 20% chloroform in acetone (AFSF), the 60% acetone in methanol (MF_1SF) and the 20% acetone in methanol (MF_2SF) were used for bioactivity studies after carrying out preliminary activity tests.

Acute toxicity study

Acute toxicity study was carried out according to the method described by Glombitza et al.[14] and Ghosh et al.[15] Overnight fasted Wistar albino rats were divided into groups, each consisting of 6 animals, and orally fed separately with the column fractions AFSF, MF_1SF and MF_2SF at dose levels of 100, 500, 1000 and 2000 mg/kg body weight, respectively. The rats were observed continuously for 2 h for behavioural, neurological and autonomic profiles. After a period of 24 and 72 h, observations were made for any death that might have occurred.[16]

Assessment of hypoglycaemic activity in euglycaemic rats

The experiment was conducted according to the procedure described in the literature.[17,18] A total of 66 normoglycaemic rats fasted for 18 h were divided into 11 groups of 6 mice per group and treated as follows: Group I - 5% gum acacia (Control group); Group II - glibenclamide, 10 mg/kg (Standard group); Group III - AFSF 100 mg/kg; Group IV - AFSF 200 mg/kg; Group V - AFSF 400 mg/kg; Group VI - MF_1SF 100 mg/kg;

Group VII - MF_1SF 200 mg/kg; Group VIII - MF_1SF 400 mg/kg; Group IX - MF_2SF 100 mg/kg; Group X - MF_2SF 200 mg/kg; Group XI - MF_2SF 400 mg/kg. Immediately before the experiment begins, initial fasting blood samples were taken from the animals of all the groups. This was followed by oral administration of different doses of the column fractions of *S. febrifuga* or the reference drug glibenclamide (10 mg/kg) suspended in 5% gum acacia. The effects of the column fractions and reference drug on fasting blood glucose level were monitored for 24 h. Blood samples were drawn from the retro-orbital plexus of the treated rats at 0 h (initial fasting blood sample) and 2, 4, 6, 8, 12 and 24 h after the treatment. The samples were analyzed on autoanalyser (Selectra Junior, Merck) for blood glucose content using glucose oxidase-peroxidase method.[19]

Assessment of antihyperglyacemic activity in alloxan-induced diabetic rats

Diabetes was induced in normoglycaemic overnight fasted Wistar albino rats by a single intraperitoneal injection of alloxan monohydrate (125 mg/kg) dissolved in saline. Blood glucose level of the animals was checked after 72 h. Animals with blood glucose level >250 mg/dl were considered diabetic and were used for the study. Diabetic rats were divided into 11 groups of six animals per group as shown above, and treated orally as described in the literature.[20,21] Blood samples were collected from all the animals at the time intervals of 0 (fasting blood sample), 2, 4, 6, 8, 12 and 24 h after treatment to estimate blood glucose levels.

Estimation of serum biochemical parameters

Serum triglycerides and serum cholesterol were estimated as described by Trinder et al.[19] Serum total proteins were measured according to the method of Lowry et al.[22] and serum insulin levels were determined by chemiluminescence assay.[23,24]

Blood samples collected from the experimental animals were subjected to centrifugation at 3000 rpm for 10 min to separate the serum. For the estimation of the above mentioned biochemical parameters, 0.5 µl of serum sample was transferred to each of the pediatric sample cups. The cups and the working reagent bottles (25 ml) corresponding to the biochemical parameters were then placed at their respective positions in the rotor system of the autoanalyzer. Thirty min after programming, each of the test parameters, the corresponding values of the different serum samples displayed on the computer were recorded.

Statistical analysis

All values were expressed as mean ± SD. The data were statistically evaluated using one way analysis of variance (ANOVA) followed by post hoc Dunnet's t-multiple comparison test using GraphPad Prism 4 computer software.[25] Values corresponding to $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Preliminary phytochemical screening revealed the presence of alkaloids, triterpenoids and saponins in AFSF, triterpenoids in MF_1SF, and alkaloids, triterpenoids and saponins in MF_2SF. In the present study, none of the fractions produced behavioural, neurological and autonomic changes in rats nor did they cause death during the first 72 h of the study period indicating that the extracts are safe at the tested doses.

Effect of fractions on blood glucose levels in euglycaemic rats

Effects of the column fractions of *S. febrifuga* bark extract on blood glucose levels in euglycaemic rats are shown in Table 1. At doses of 200 and 400 mg/kg, AFSF produced a significant ($P < 0.05$) hypoglycaemic effect 2 h after treatment, while the same doses of MF_1SF and MF_2SF showed similar effects 4 h after treatment. All fractions showed significant hypoglycaemic effect at all test doses with a P value < 0.01 at 4, 6 and 8 h of the study. AFSF (200 mg/kg) and the reference drug glibenclamide (10 mg/kg) exhibited significant effect with P value < 0.05, 2 h after treatment. Maximum percentage reduction in blood glucose levels ranging from 34.4 to 54.1 was obtained 6 h after administration of the fractions. However, maximum hypoglycaemia of 54.1, 43.0 and 37.8% was observed 6 h after administration of 200 mg/kg of AFSF, MF_2SF and MF_1SF, respectively. Percentage reduction of blood glucose level by the 200 mg/kg of AFSF (54.1) was comparable to that of the reference drug, glibenclamide (10 mg/kg; 54.8).

Effect of fractions on fasting blood glucose levels in alloxan-induced diabetic rats

Effect of the column fractions obtained from *S. febrifuga* bark extract on blood glucose levels of alloxan-induced diabetic rats is shown in Table 2. At doses of 100, 200 and 400 mg/kg, all the fractions significantly ($p < 0.01$) lowered blood glucose levels 2 h after treatment, and the effect continued up to 24 h of the study period. However, all the fractions showed maximum effect, 6 h after treatment with the highest effect observed for the

Table 1. Effect of column fractions obtained from the bark extract of *Soymida febrifuga* on blood glucose levels in euglycaemic rats.

			Blood glucose level (mg/dl)						
			0 h	2 h	4 h	6 h	8 h	12 h	24 h
I	Control	—	80.5 ± 9.51	80.6 ± 7.62 (0.12%)	78.4 ± 8.01 (2.60%)	78.3 ± 9.10 (2.73%)	79.3 ± 8.01 (1.49%)	77.5 ± 8.29 (3.70%)	80.8 ± 8.08 (0.37%)
II	Gliben-clamide	10	84.1 ± 8.06	60.7 ± 3.50 (27.82%)*	51.5 ± 6.20 (38.70%)**	38.0 ± 6.10 (54.80%)**	48.5 ± 8.40 (42.30%)**	61.3 ± 8.00 (27.10%)**	73.5 ± 7.20 (12.60%)
III	AFSF	100	90.2 ± 20.1	67.9 ± 10.10 (24.70%)	60.3 ± 11.50 (33.20%)**	48.8 ± 10.90 (45.90%)**	54.6 ± 10.30 (39.47%)**	63.6 ± 10.10 (29.50%)**	74.6 ± 13.52 (5.30%)
IV	AFSF	200	85.6 ± 7.90	61.7 ± 5.90 (27.90%)*	53.9 ± 5.60 (37.00%)**	39.3 ± 7.10 (54.10%)**	51.1 ± 7.30 (39.10%)**	62.8 ± 7.30 (26.60%)**	73.7 ± 6.90 (13.90%)
V	AFSF	400	85.0 ± 8.20	62.9 ± 5.80 (26.00%)*	53.6 ± 8.60 (36.90%)**	40.8 ± 7.10 (52.00%)**	50.8 ± 6.00 (40.20%)**	63.2 ± 7.30 (25.60%)**	79.6 ± 6.90 (6.35%)
VI	MF_1SF	100	85.5 ± 13.90	71.6 ± 12.30 (16.25%)*	66.6 ± 12.21 (22.10%)**	55.6 ± 12.20 (34.90%)**	63.5 ± 10.30 (25.70%)**	70.5 ± 9.10 (17.50%)**	76.5 ± 11.90 (10.50%)
VII	MF_1SF	200	85.0 ± 8.39	66.8 ± 11.30 (21.40%)*	61.5 ± 8.50 (27.60%)**	44.8 ± 7.40 (47.30%)**	56.8 ± 6.80 (33.10%)**	66.5 ± 7.50 (21.70%)**	74.1 ± 6.50 (12.90%)
VIII	MF_1SF	400	86.5 ± 10.53	67.9 ± 8.10 (21.50%)*	59.9 ± 10.70 (30.60%)**	46.3 ± 8.90 (45.90%)**	59.5 ± 8.60 (31.20%)**	68 ± 8.10 (21.30%)**	81.6 ± 8.50 (5.60%)
IX	MF_2SF	100	86.4 ± 13.40	70.6 ± 12.12 (18.20%)*	67.3 ± 10.10 (22.40%)**	56.6 ± 12 (34.40%)**	64.5 ± 11.30 (25.30%)**	70.3 ± 11.10 (18.90%)**	77.3 ± 12.10 (10.80%)
X	MF_2SF	200	85.3 ± 10.20	65.5 ± 11.20 (22.90%)*	59.3 ± 8.90 (30.50%)**	48.55 ± 7.80 (43.00%)**	59.3 ± 10.80 (30.50%)**	65 ± 6.50 (23.50%)**	79.8 ± 19.00 (6.40%)
XI	MF_2SF	400	85.0 ± 8.21	66.4 ± 10.80 (21.60%)*	60.4 ± 6.31 (28.90%)**	50.67 ± 10.10 (41.00%)**	60.9 ± 9.70 (28.30%)**	65.9 ± 7.30 (22.40%)**	80.2 ± 9.50 (5.60%)

AFSF = 20% chloroform in acetone fraction; MF1SF = 60% acetone in methanol fraction; MF2SF = 20% acetone in methanol fraction; All values are expressed as mean + SD, n = 6; Figures in parenthesis indicate the percentage reduction of blood glucose levels when compared to 0 h value(s), *p < 0.05; **p < 0.01 when compared with control at the respective time interval.

Table 2. Effect of column fractions obtained from the bark extract of *Soymida febrifuga* on blood glucose levels in alloxan-treated rats.

			Blood glucose levels (mg/dl) at different hours						
			0 h	2 h	4 h	6 h	8 h	12 h	24 h
I	Control	—	279.4 ± 2.5	280.4 ± 2.3 (0.35%)	281.3 ± 3.1 (0.68%)	281.4 ± 6.1 (0.72%)	281.8 ± 3.1 (0.85%)	280.4 ± 3.2 (0.35%)	280.6 ± 2.8 (0.43%)
II	Gliben-clamide	10	280.3 ± 1.6	235.0 ± 4.8 (16.60%)*	214.0 ± 5.0 (23.65%)*	186.5 ± 3.2 (33.46%)*	198.3 ± 4.2 (29.25%)*	216.0 ± 9.3 (22.90%)*	239.1 ± 14.2 (14.69%)*
III	AFSF	100	280.4 ± 2.1	240 ± 1.6 (14.40%)*	219.0 ± 2.1 (21.8%)*	190.8 ± 2.2 (31.90%)*	200.0 ± 2.3 (28.60%)*	221.5 ± 3.2 (21.00%)*	242.4 ± 2.3 (13.50%)*
IV	AFSF	200	280.5 ± 3.9	236.5 ± 3.2 (15.60%)*	215.3 ± 4.5 (23.30%)*	187.7 ± 3.3 (33.08%)*	199.4 ± 3.2 (28.90%)*	217.6 ± 4.3 (22.40%)*	239.9 ± 5.8 (14.40%)*
V	AFSF	400	284.3 ± 3.5	253.0 ± 3.5 (11.00%)*	229.8 ± 2.3 (19.16%)*	196.6 ± 4.8 (30.84%)*	214.5 ± 4.2 (24.55%)*	220.5 ± 2.5 (22.35%)*	253.5 ± 6.3 (10.80%)*
VI	MF_1SF	100	280.6 ± 2.4	250.6 ± 4.3 (10.70%)*	225.0 ± 7.8 (19.80%)*	199.6 ± 9.3 (28.80%)*	204.4 ± 8.5 (27.20%)*	229.6 ± 8.8 (18.10%)*	246.6 ± 8.8 (12.10%)*
VII	MF_1SF	200	280.5 ± 1.4	240.2 ± 4.01 (14.40%)*	220.4 ± 4.8 (21.40%)*	190.6 ± 5.1 (32.00%)*	202.4 ± 8.3 (27.80%)*	221.0 ± 10.3 (21.20%)*	243.3 ± 10.3 (13.30%)*
VIII	MF_1SF	400	282.3 ± 1.6	258.1 ± 3.6 (8.60%)*	214.9 ± 2.1 (23.80%)*	199.9 ± 5.6 (29.10%)*	215.3 ± 4.6 (23.75%)*	223.3 ± 4.6 (20.90%)*	245.0 ± 3.8 (13.50%)*
IX	MF_2SF	100	280.3 ± 1.5	255.4 ± 4.1 (8.70%)*	227.1 ± 6.8 (18.90%)*	198.3 ± 3.6 (29.20%)*	205.3 ± 5.9 (26.70%)*	227.5 ± 8.3 (18.70%)*	247.5 ± 5.8 (11.60%)*
X	MF_2SF	200	281.4 ± 1.6	242.1 ± 2.9 (13.80%)*	221.4 ± 4.3 (21.30%)*	189.6 ± 5.8 (32.50%)*	200.7 ± 8.5 (28.50%)*	223.5 ± 10.4 (20.40%)*	244.4 ± 10.2 (13.10%)*
XI	MF_2SF	400	282.5 ± 1.5	256.2 ± 3.5 (9.30%)*	216.40 ± 1.8 (23.39%)*	199.4 ± 5.9 (29.50%)*	215.5 ± 3.6 (23.70%)*	220.5 ± 4.6 (21.94%)*	249.5 ± 2.8 (11.60%)*

AFSF = 20% chloroform in acetone fraction; MF_1SF = 60% acetone in methanol fraction; MF_2SF = 20% acetone in methanol; All values are expressed as mean ± SD, n = 6; Figures in parenthesis indicate the percentage reduction of blood glucose levels when compared to 0 h value(s); *P < 0.01 when compared with control at the respective time interval.

200 mg/kg of AFSF, MF_2SF and MF_1SF, which showed 33.0, 32.5 and 32.0% reduction of blood glucose levels, respectively. Although significant blood glucose lowering effect was also achieved by the 400 mg/kg of each of the fractions, 6 h after treatment, the reduction was much less than that of the lower dose (200 mg/kg). The significant ($p < 0.01$) antihyperglycaemic effect achieved by the 200 mg/kg of AFSF was comparable to that of the

reference drug glibenclamide (10 mg/kg) at any time interval of the study period, with maximum percent reduction of 33.0 and 33.46, respectively.

Effect of fractions on body weight of alloxan-induced diabetic rats

The effect of the fractions on body weight of alloxan-induced diabetic rats study is summarized in Table 3. The observations made on the results of different parameters studied are as follows: There was a gradual decrease in body weight of animals in diabetic control group. Animals treated with fractions and reference drug showed a gradual and significant ($p < 0.01$) increase in body weight after 7 days of the treatment. The increase in body weight was observed until the end of the study period (21 days). The significant ($p < 0.01$) effect of all the fractions at a dose of 200 mg/kg on body weight was comparable to that of the reference drug, glibenclamide (10 mg/kg) at each time interval of the study.

A significant reduction in body weight observed in alloxan-induced diabetic rats was very likely due to increased excretion of glucose and reduced uptake of glucose by peripheral tissues and glycogen synthesis. The improvement in body weight of the animals when treated with the fractions is possibly due to potentiation of insulin secretion which reverses these effects. A significant reduction in body weight was observed in alloxan-induced diabetic rats. The decrease is likely due to increased excretion of glucose and reduced uptake of glucose by peripheral tissues and glycogen synthesis.[26] Improvement in body weight of the animals when treated with the fractions indicates a protective effect.

Effect of fractions on serum biochemical parameters in alloxan-induced diabetic rats

Results of the effects of column fractions on the various biochemical parameters are shown in Table 4.

Serum triglyceride level

In groups treated with fractions and reference drug, a significant ($p < 0.001$) decrease in serum triglyceride level was recorded at day 21 the study period. The percent reduction in serum triglyceride level in AFSF, MF_1SF and MF_2SF treated groups were 12.18, 12.55 and 12.26, respectively, whilst glibenclamide (10 mg/kg, b.w.)

Table 3. Effect of column fractions obtained from the bark extract of *Soymida febrifuga* on body weight in alloxan-induced diabetic rats.

		Body weight in grams			
		Day 1	Day 7	Day 14	Day 21
Diabetic control-I	–	220.6 ± 6.20	207.5 ± 9.40	190.4 ± 8.50	179.0 ± 5.90
Glibenclamide-II	10	219.3 ± 10.80	230.2 ± 9.03**	244.4 ± 8.90**	256.3 ± 10.80**
AFSF-III	200	219.9 ± 10.60	229.0 ± 8.90**	242.4 ± 5.30**	250.4 ± 8.30**
MF_1SF- IV	200	218.4 ± 11.50	225.8 ± 7.60*	238.4 ± 6.50**	246.5 ± 5.60**
MF_2SF -V	200	219.5 ± 10.60	227.6 ± 1.80**	239.0 ± 7.50**	249.5 ± 5.80**

AFSF = 20% chloroform in acetone fraction; MF_1SF = 60% acetone in methanol fraction; MF_2SF = 20% acetone in methanol fraction;
All values are expressed as mean ± SD, n = 6; Values given in the parenthesis are percent blood glucose reduction when compared to 1st day value(s); * P < 0.01; ** P < 0.001 when compared with control at the respective time interval.

Table 4. Effect of column fractions obtained from the bark extract of *Soymida febrifuga* on different serum biochemical parameters in alloxan-induced diabetic rats.

		Serum insulin levels in µIU/ml		Serum triglyceride levels (mg/dl)		Serum cholesterol (mg/dl)		Serum total protein (g/dl)	
		Day 1	Day 21	Day 1	Day 21	Day 1	Day 21	Day 1	Day 21
Diabetic control - I	–	10.7 ± 0.6	10.9 ± 0.60 (1.86%)	180.8 ± 1.50	181.2 ± 1.80 (0.22%)	122.4 ± 6.00	122.9 ± 3.50 (0.40%)	4.2 ± 0.5	4.3 ± 0.40 (0.70%)
Glibenclamide - II	10	11.2 ± 0.6	18.9 ± 1.90 (68.75%)	172.5 ± 2.90	152.3 ± 3.00** (11.70%)	121.2 ± 4.30	67.5 ± 10.30** (44.30%)	3.9 ± 0.2	5.8 ± 0.70** (48.70%)
AFSF- III	200	11.3 ± 0.3	19.0 ± 2.20** (68.10%)	171.5 ± 2.60	150.6 ± 2.60** (12.18%)	121.3 ± 3.20	71.4 ± 8.30** (41.13%)	4.1 ± 0.2	6.0 ± 0.70** (65.34%)
MF_1SF - IV	200	11.4 ± 0.4	19.1 ± 2.80** (64.60%)	170.5 ± 4.80	149.1 ± 3.90** (12.55%)	120.5 ± 4.04	72.1 ± 8.40** (40.16%)	4.4 ± 0.3	5.7 ± 0.50** (43.18%)
MF_2SF - V	200	11.3 ± 0.6	19.4 ± 4.0** (71.68%)	174.6 ± 4.90	153.2 ± 4.20** (12.26%)	120.1 ± 3.61	72.1 ± 7.40** (39.96%)	4.4 ± 0.3	5.3 ± 0.32* (41.14%)

AFSF = 20% chloroform in acetone fraction; MF_1SF = 60% acetone in methanol fraction; MF_2SF = 20% acetone in methanol fraction; All values are expressed as mean ± SD, n = 6; Values given in the parenthesis are the percent increase or decrease in respective parameter level; * P < 0.05; ** P < 0.001 when compared with control at the respective time interval.

reduced serum triglyceride level by 11.7% in the positive control group.

Serum cholesterol level

At a dose of 200 mg/kg, all the fractions significantly ($p < 0.001$) lowered serum cholesterol level at day 21 of the study period. The percent reduction in serum cholesterol level in AFSF, MF_1SF and MF_2SF groups were 41.13, 40.16 and 39.96, respectively. The percent reduction of glibenclamide was 44.3. The statistically significant ($p < 0.01$) antihypercholesterolemic effect of the fractions was comparable to that of the reference drug.

Serum total protein level

At a dose of 200 mg/kg, all the fractions showed a significant increase in serum total protein level by 65.0, 43.18 and 41.14%, respectively. In glibenclamide treated group, the increase was 48.7%. Though the statistically significant effect of the fractions and reference drug were comparable to each other, the effect of AFSF was greater than that of the other two fractions and glibenclamide.

Serum insulin

In AFSF, MF_1SF and MF_2SF groups, a significant ($p < 0.001$) increase in serum insulin level was observed after 21 days. The percent increase in serum insulin level was 68.1 and 64.6 and 71.8 for AFSF, MF_1SF and MF_2SF, respectively, which was very close to the effect shown by the reference drug, glibenclamide.

Several plants with proven hypoglycaemic effects have been reported to contain compounds like terpenoids[27], glycosides[28,29], alkaloids[30] and saponins.[31] Preliminary phytochemical screening indicated the presence of one or more of these classes of compounds in the fractions studied. The observed hypoglycaemic effect may be a result of individual compound or a combination of these constituents. The hypoglycaemic activity exhibited by these fractions could be due to one or more of the following. The fractions may: (1) potentiate pancreatic secretion of insulin from β-cell of islets of Langerhan's (2) simulate uptake of glucose by peripheral tissues (3) inhibit endogenous glucose production (4) stimulate gluconeogenesis in liver and muscles.

There is an association between diabetes and hyperlipidemia. Insulin is responsible for activation of the lipolytic enzyme lipoprotein lipase which hydrolyses triglyceride under normal conditions. Therefore, destruction of β-cells results in decreased plasma insulin and ultimately hyperlipidemia. Significant control of plasma lipid levels by the tested fractions suggests that they produce the action by possibly improving secretion of insulin.[32] The effect of the fractions on cholesterol and triglyceride levels may be due to decrease in activity of enzymes involved in cholesterol biosynthesis or low levels of lipolysis which are in turn controlled by insulin.[33] The above findings confirm that the tested fractions have antidiabetic activity and are capable of correcting the altered biological parameters.

CONCLUSION

As the percentage of diabetic toll increases day by day, there is an urgent need for natural antidiabetics and hypoglycaemic agents to be explored. In the present study, the various column fractions obtained from the bark extract of *S. febrifuga* showed significant hypoglycaemic and antihyperglycaemic activites in normal healthy and alloxan-induced diabetic rats, respectively. At a dose of 200 mg/kg, the 20% chloroform in acetone eluate showed the maximum activity that was comparable to that of glibenclamide. It can therefore be concluded that there is a possibility of getting effective compounds from the bark extract of *S. febrifuga*, which can be of value in the fight against diabetes. Needless to say that further *in vivo* activity and chronic toxicity studies are required before determining the possible therapeutic value of the plant.

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Comparative *in vitro* antioxidant evaluation of different extracts of *Camellia sinensis* leaves from different geographical locations

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ABSTRACT

The present study was conducted to assess and compare the *in vitro* antioxidant property of four different solvent extracts from *Camellia sinensis* (tea) leaves collected form five different geographical regions in India. All the extracts at two different concentrations (500 and 1000 µg/ml) was tested for their *in vitro* free radical scavenging potential by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay. All the test extracts were found to exhibit remarkable free radical scavenging or antioxidant effect. The tea sample form Kerala was found to be the most active. The differential effect was plausibly due to geographical variations in chemical constituents especially polyphenol contents of *C. sinensis* leaf.

Keywords: *Camellia sinensis*, antioxidant, polyphenols, leaf.

INTRODUCTION

Camellia sinensis L. (Theaceae), commonly known as tea, is a large evergreen shrub indigenous to Eastern Asia where it is cultivated extensively. Tea is actually a product made from leaf and bud of the plant, *C. sinensis*, and is the second most consumed beverage in the world.[1] The dried cured leaves of *C. sinensis* have been used to prepare beverages for more than 4000 years. The method of curing determines the nature of the tea to be used for infusion. Green tea is a type of cured tea that is 'non fermented' and produced by drying and steaming the fresh leaves; whereas black tea leaves are withered, rolled, fermented and then dried.[2] *C. sinensis* leaf has been used medicinally for centuries in traditional Chinese medicine (TCM). Recently there has been renewed interest in green tea because of its role in the prevention of several disease risks and other important health benefits.[3] Previous researchers have reported several pharmacological and toxicological properties of *C. sinensis* leaf on animals and humans.[4,5] The present study was conducted to assess and compare the *in vitro* antioxidant property of extracts from tea leaves collected from different geographical regions of India.

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MATERIALS AND METHODS

Plant material

The mature green leaves of *Camellia sinensis* L. (Theaceae) were collected in August 2008 from five different locations in India. The leaves were sourced from Darjeeling (West Bengal), Guwahati (Assam), Coonoor (Tamil Nadu), Coorg (Karnataka) and Munnar (Kerala) regions of India. Just after collection, the tea leaves were shade dried at room temperature (24–26°C) and ground mechanically into a coarse powder.

Extraction

The powdered leaves of green tea (40 g) were extracted separately with different solvents namely ethyl acetate,

Table 1. Different solvent extractive values of *C. sinensis* leaves from different locations.

Solvents	% Extractives				
	Assam	Darjeeling	Tamil Nadu	Karnataka	Kerala
Ethyl acetate	16.44	19.23	14.66	12.98	17.00
Methanol	22.09	34.12	24.86	25.94	26.52
Ethanol	30.41	49.37	25.22	28.37	30.99
Aqueous methanol	33.47	32.71	26.93	35.22	31.48

ethanol, methanol and aqueous methanol (60:40) by boiling under reflux for 8 h. All the extracts were concentrated by distilling off the solvents at low temperature using vacuum. The dry extracts were then weighed and the percentage of different extractive values was calculated with respect to the air dried powdered plant material. The results are presented in Table 1.

Chemicals

L-ascorbic acid (vitamin C) and 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade obtained commercially.

Evaluation of *in vitro* antioxidant activity

The antioxidant or free radical scavenging activity of all of the extracts was measured by 1, 1-diphenyl-2-picryl-hydrazil (DPPH) using the reported method.[6] Briefly, a 0.1 mM solution of DPPH in methanol was prepared, and 1 ml of this solution was added to 3 ml of the all of the extract solutions, in petroleum ether, chloroform and methanol at two different concentrations (500 and 1000 µg/ml), respectively. Ascorbic acid at a concentration of 500 µg/ml served as reference. The mixture was shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-Vis spectrophotometer. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1) / A_0) \times 100]$$

where, A_0 is the absorbance of the control, and A_1 is the absorbance in presence of all of the extract samples and reference agent. The results are summarized in Table 2.

RESULTS AND DISCUSSION

Free radicals are molecules containing one or more unpaired electrons in atomic or molecular orbitals. There is increasing evidence that aberrant production of free radicals *in vivo* leads to increased oxidative stress on cellular structures and causes changes in molecular pathways that underpins the pathogenesis of several important diseases, including carcinogenesis and cancer, cardiovascular diseases, neurodegenerative diseases and in the process of physiological ageing. Agents that can scavenge the reactive free radicals are known as antioxidants.[7]

The stable 1, 1-diphenyl-2-picryl-hydrazil (DPPH) radical model is a widely used, relatively quick and precise method for the evaluation of free radical scavenging or antioxidant activity. The effects of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen-donating ability. DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The absorption maximum of a stable DPPH radical in methanol was 517 nm. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progressed, results in the scavenging of the radicals by hydrogen donation. It is visually noticeable as a change in colour from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the antioxidant activity of different antioxidant agents.[8,9]

Table 2. Antioxidant property different extracts of *C. sinensis* leaves from different locations.

Extracts	Concentration (µg/ml)	% DPPH scavenging					
		Darjeeling	Kerala	Assam	Karnataka	Tamil Nadu	Ascorbic acid
Ethyl Acetate	500	54.85	51.59	55.08	54.56	51.12	72
	1000	61.28	68.66	62.13	66.49	61.28	
Methanol	500	54.91	53.72	53.83	50.73	51.71	
	1000	68.05	73.80	68.91	62.88	68.05	
Ethanol	500	56.27	50.99	56.52	56.29	50.57	
	1000	65.43	61.18	65.48	66.22	65.43	
Aq. methanol	500	56.10	53.22	58.15	52.53	52.15	
	1000	69.82	73.07	73.05	61.48	69.82	

Based on the data obtained from the present study, all the test extracts were found to demonstrate markedly effective *in vitro* free radical scavenging or antioxidant property. The results are summarized in the Table 2. In all the cases, the free radical scavenging activity was concentration dependent i.e., increased with increasing concentration form 500 to 1000 µg/ml. The effects were comparable to that of reference agent ascorbic acid (500 µg/ml). The tea sample form Kerala was found to be the most active. The aqueous methanol (60:40) extracts of all five samples were found to be most effective in all cases. This indicated aqueous methanol extracted maximum antioxidative principles (presumably polyphenols) form *C. sinensis* leaf.

Tea leaves contain varying amounts of polyphenols particularly flavonoids. Polyphenols are well known natural products known to possess several notable biological properties including excellent antioxidant activity.[10] The main flavonoids present in green tea leaves include catechins (flavan-3-ols) and most importantly epigallocatechin-3-gallate (EGCG).[11] Tea leaf also contains some phenolic acids such as chlorogenic acid, gallic acid, caffeic acid, etc. which also have antioxidant effects.[12] The observed differences in antioxidant activity of tea leaf extracts may be attributed to the variations of composition in the polyphenol contents of *C. sinensis* leaves grown in different geographical locations due to environmental or climatic factors.

The present preliminary study confirms remarkable *in vitro* free radical scavenging activity of four different solvent extracts of *C. sinensis* leaf collected from five different geographical locations against DPPH. All the leaf extracts demonstrated marked antiradical property. *C. sinensis* leaf collected form Kerala was found to be the most active. The differential antioxidant effect was plausibly due to geographical variations in chemical constituents especially polyphenol content of *C. sinensis* leaf.

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Polyphenolic constituents of the methanolic extract of *Callistemon viridiflorous* leaves and its antimicrobial activity

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ABSTRACT

Introduction: The genus *Callistemon* (family: Myrtaceae) contains 34 species which are widely distributed in the temperate regions and used in folk medicine. **Methods:** Chromatographic separation of 80% MeOH extract of the leaves of *Callistemon viridiflorus (sims) Sweet* (*Cv*) was performed. **Results:** Seven known polyphenolic compounds were isolated for the first time from this species: gallic acid, ellagic acid, isoquercetin, hyperin, 1,2:3,4-(bis(s)-hexahydroxy diphenoyl-β-D-glucopyranose, nilocitin and quercetin-3-O-α-L-glucuronopyranoside. The methanolic extract of *Cv* leaves exhibited a significant anti-microbial activity against the tested microorganisms.

Keywords: Myrtaceae, *Callistemon viridiflorous*, polyphenols, anti-microbial activity.

INTRODUCTION

Family Myrtaceae (Myrtle family) comprises about 130–150 genera and about 5000 species of evergreen shrubs and trees. The genus *Callistemon* (family: Myrtaceae) contains 34 species which are widely distributed in the temperate regions of Australia, and are widely distributed in warm-temperate regions.[1–5] The genus *Callistemon* (commonly named bottle brush plant) is known in folk medicine for its anticough, antibronchitis, antifungal, antibacterial, anti-inflammatory, analgesic, anticonvulsant, antidiabetic, anti-hemorrhoidal and antinociceptive activities.[6–13] Previous phytochemical investigations of some species of *Callistemon* genus resulted in the identification of *C*-methyl flavonoids, falvonol glycosides, phenolic acids, hydrolysable ellagitannins, triterpenoids and phloroglucinol derivatives.[4–19] Because of the biological importance of plant polyphenols, particularly tannins and flavonoids, a phytochemical study was of interest to investigate the constitutive poyphenols in the extract of leaves for *Callistemon viridiflorous*. Synonyms of the plant include *Callistemon viridiflorus* (Sieber ex Sims) Sweet, *Callistemon salignus* var. *viridiflorus* (Sieber ex Sims) F. Muell., *Melaleuca virens* Craven, *Metrosideros viridiflora* Sieber ex Sims.[20]

The present study deals with the isolation and identification of some polyphenolic constituents of *Callistemon viridiflorous* species growing in Egypt and to explore the antimicrobial activity of its methanolic extracts.

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MATERIALS

Plant material

Leaves of *Callistemon viridiflorus* (Sims) Sweet were collected from Alexandria-Cairo Road, Egypt, in July, 2008. The plant was identified by Dr. Trease Labeb, Lecturer of Taxonomy, Department of Floral and Taxonomy, Orman Garden, Cairo, Egypt.

Microorganisms

Gram-positive bacteria (*Staphylococcus aureus* ATCC4175), Gram-negative bacteria (*E. coli*) and yeast (*candida albicans*

ATCC60193) were supplied by Microbiology Department, Faculty of Pharmacy, Helwan University.

Culture media

Trypticase soy agar (Difco) was used as a culture media.

Standard antimicrobial agents

Ofloxacin and Ketoconazol were used as positive control.

Chemicals, sugars, drugs and authentic reference materials for chromatography

Galactose, glucuronic acid, glucose, quercetin, hyperin, isoquercetin, kaempferol, ellagic acid, gallic acid and nilocitin were obtained from Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Helwan, Egypt. Polyamide S6 (50–160 µm, Fluka chemie AG, Switzerland) for column chromatography. Microcrystalline cellulose (E. Merck, Darmstadt, Germany) for column chromatography. Sephadex LH-20 (25–100 µm, Pharmacia, Uppsala, Sweden) for column chromatography. Silica gel 60 F_{254} precoated aluminum sheets (20 × 20, 0.2 mm thickness) and cellulose precoated aluminum sheets (20 × 20, 0.2 mm thickness), (E. Merck, Darmstadt, Germany) for thin layer chromatography. Whatmann No.1 for paper chromatography (Whatmann Ltd., Maidstone, Kent, England). Diphenyl borinic acid ethanolamine complex (Naturstoff reagent for flavonoids).[40] Aluminum chloride reagent (1% in ethanol) for flavonoids.[21] Ferric chloride reagent (1% in ethanol) for phenolic compounds.[22] Aniline hydrogen phthalate reagent for sugars.[23] Nitrous acid spray reagent for ellagitannins.[24] Potassium iodate spray reagent for gallotannins,[25] Reagents for UV spectroscopic analysis of flavonoids such as sodium methoxide, Aluminium chloride, HCl, Boric acid and sodium acetate.[26]

Solvent systems

S_1	*n*-Butanol – Acetic acid –Water (BAW)	(4:1:5 v/v/v, top layer)
S_2	Acetic acid – Water	15:85 v/v
S_3	*n*-Butanol – Isopropyl alcohol – Water	(4:1:5 v/v/v, top layer)

Apparatus

NMR spectrometers: JOEL GX-500 (500 and 125 MHz for ^1H and ^{13}C NMR), NMR Department, National Research Center (NRC) were used. The results were reported as δppm values relative to TMS as internal reference. All samples have been prepared in DMSO-d_6 solvent. MS Spectrometer: Finnigan LCQ-deca or Finnigan LTQ for HRESI-MS, Strathclyde Institute of Pharmacy and Biomedical Science, Glasgow, United Kingdom. UV-visible spectrophotometer: JASCO (V-630) UV spectrophotometer was used for UV spectroscopic analysis of isolated compounds in UV range (200–450 nm) in different diagnostic shift reagents, Faculty of Pharmacy, Helwan University. Rotary evaporator (Bűchi, G, Switzerland). Ultraviolet lamp (VL-215 LC, Marne La Vallee, France) was used for visualization of spots on paper and thin layer chromatograms and follow up the columns fractionation on columns at 254 and/or 365 nm.

METHODS

Extraction and isolation of polyphenolic constituents from *Callestimon virdiflorous* leaves

The air dried ground leaves (1500 g) were subjected to exhaustive extraction with hot 80% aq. methanol under reflux (70° C). The collective extract was dried under vacuum (45° C) and resulted in 240 g dry extract. The dried extract was treated by petroleum ether under reflux (60° C). The pet-ether unsoluble portion was suspended in water and preliminary fractionated on polyamide column using a step gradient H_2O/MeOH mixture with decreasing polarity from 100% water to MeOH 100% for elution to yield 35 individual fractions. Those fractions were collected into five major fractions, which were subjected to successive column chromatography on cellulose, sephadex LH-20. The eluted fractions were tested by spotting using two dimensional paper technique (2D-PC), S_1 used for the first run and S_2 for second run. After air drying, the spots were visualized under UV-light before and after spraying with a suitable reagent.[23,26] Glass columns of different sizes were packed with required adsorbent. Methanol soluble part of fraction 1 was applied on Sephadex-LH_{20} column and eluted by methanol resulted in compound F_1. Fraction 2 was applied on Sephadex-LH20 column and eluted by 20% H2O/80% methanol followed by ethanol resulted in compound F_2. Fraction 3 was applied on cellulose (20% MeOH/H_2O) followed by sephadex LH-20 (eluted by S_3 solvent followed by 30% EtOH/H_2O) resulted in compound F_3 and F_4. Fraction 4 was applied on cellulose column and eluted by S_3 solvent followed by 20–60% MeOH/H2O then subfractions applied on Sephadex-L20 column and eluted by MeOH which resulted in compound F_{6a} and F_{6b}. Fraction 5 will be discussed later. Bands formed during the elution process were traced in both visible and UV light.

Each fraction eluted from the column was evaporated under reduced pressure and analyzed by PC or TLC; similar fractions were collected and separately investigated. About 2–3 mg of each glycoside was treated with 0.1 N HCl in methanol for 60 min at 100° C. The reaction mixture was examined every 10 min by CoPC to detect any intermediate or sugars that may be formed against the available authentic samples.[27] A complete acid hydrolysis was carried out by treating few mgs of each glycoside with 1.5N HCl in aqueous 50% methanol for 2 hr at 100° C, then evaporated and extracted with ethyl acetate. Aglycones were traced in ethyl acetate fraction by CoPC alongside authentic aglycones or by using different spectroscopic analysis. The aqueous phase was neutralized with sodium bicarbonate (5% aqueous solution) and used for investigation of the sugar moiety on CoPC against authentic sugar samples and spraying with aniline hydrogen phthalate reagent.[27] Few spicks of each pure hydrolysable tannin were heated at 100° C under reflux for about 8 hr with 2 N H_2SO_4 in 50% aqueous methanol. After cooling the aqueous phase was extracted with ethyl acetate, then filtered on anhydrous sodium sulphate and concentrated under vacuum. This was used for identification of phenolic acids on PC against authentic samples and using S_1 and S_2 as solvent systems, whereas the neutralized aqueous phase with 0.5% $NaHCO_3$, was examined for the identification of the present sugars, using CoPC.[27]

Microbiological study

The disc agar diffusion method

The antimicrobial study was determined using modified Kirby-Bauer disc diffusion method.[28] One hundred ml of the test bacteria or fungi were grown in 10 µl of fresh media until they reached a count of approximately 108 cells/ml. 100 ml of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained. Disc diffusion method for yeasts developed by using approved standard method (M44-P) by the NCCLS.[41] Plates with Gram +ve bacteria as *Staphylococcus aureus*; Gram -ve bacteria as *Escherichia coli* were incubated at 35–37° C for 24–48 hours. Yeast as *Candida albicans* was incubated at 30° C for 24–48 hours then the diameters of the inhibition zones were measured in millimeters. The extract of *Cv* leaves were diluted with DMSO, 1:5 w/v, then 20 µl (4 µl of diluted extract/disc) was aseptically transferred onto sterile discs of Whatman filter paper (5 mm diameter). Standard discs of ofloxacin and fluconazole served as positive controls for antimicrobial activity.

RESULTS AND DISCUSSION

Characterization and identification of isolated compounds

Compound F$_1$ Yellowish white amorphous powder (40 mg). chromatographic properties: R_f value: 0.71 (S_1), 0.56 (S_2); shine violet fluorescent under UV-light turned to deep blue color with $FeCl_3$ spray reagent. ^{1}HNMR (500 MHz, DMSO-d6), δppm 6.87 (2H, S, H-2/6). ^{13}CNMR (125 MHz, DMSO-d6), δppm 168.13 (C-7), 145.86 (C-3/5), 138.52 (C-4), 120.99 (C-1), 109.24 (C-2/6). From the previous data and by comparison with previous reported data.[29,39] Compound F_1 was confirmed as gallic acid which was isolated for the first time from *C. viridiflorus* and identified earlier from the leaves of *C. lanceolatus*.

Compound F$_2$ Bright yellow amorphous powder (60 mg). chromatographic properties: R_f values; 0.46 (S_1), 0.11 (S_2); buff fluorescent under long and short UV-light turned to dull yellow with ammonia vapors and greenish yellow with Naturstoff reagent. It gave faint blue color with $FeCl_3$ reagent. ^{1}HNMR (500 MHz, DMSO-d6), δppm 7.49 (2H, s, H-5/5`). $^{13}$ CNMR (125 MHz, DMSO-d6), δppm 159.73 (C-6/6`), 148.72 (C-4/4`), 140.48 (C-3/3`), 136.89 (C-2/2`), 112.93 (C-1/1`), 110.71 (C-5/5`). According to the previous data and by comparison with the previous reported data.[29–30] Compound F_2 was confirmed as ellagic acid which was identified earlier from *C. lanceolatus* and first time from *C. viridiflorus*.

Compound F$_3$ Brown amorphous powder (35 mg). Chromatographic properties: R_f values; 0.42(S_1), 0,81(S_2), dark purple spot under short and long UV light, it gave blue color with $FeCl_3$ reagent and rose red colour, colour changed to dark brown with HNO_2 reagent (specific spray reagent for ellagitannins).[43] On complete acid hydrolysis it afforded ellagic acid in organic phase and glucose in aqueous phase (CoPC). ^{1}HNMR (500 MHz, DMSO-d6), δppm 6.62, 6.50, 6.48, 6.33 (each 1H, s, H- 6````/6```/6``/6`, 2 x HHDP), 5.45 (1H, dd, *J*= 9.5, 10.0 Hz,H-2), 5.35 (1H, d, *J*= 9.5Hz, H-1), 4.84 (1H, t, *J*= 10.0 Hz, H-3), 4.74(1H, t, J=10.0 Hz, H-4), 3.91(1H, brd, *J* = 11.85 Hz, H-6). ^{13}CNMR (125 MHz, DMSO-d6), δppm 168.66, 166.30, 165.86, 163.38 (C-7````/7```/7``/7`, 4x carbonyl HHDP), 146.96, 145.41, 145.21, 145.01 (4 x C-3 HHDP), 144.94, 144.60, 144.18, 144.17 (4 x C-5 HHDP), 137.57, 136.16, 136.02,135.49 (4 x C-4 HHDP), 126.32, 125.30, 125.20, 123,96(4 x C-1HHDP), 115.63, 115.59, 114.89, 114.75(4 x C-2 HHDP), 107.32, 107.22,

105.99, 105.98 (4 x C6 HHDP), 93.60(C-1), 79.22(C-3), 73.25(C-2), 70.35(C-5), 68.36(C-4), 65.60(C-6). Negative ESI-MS, m/z 784.41[M-H] , 482.5[M- HHDP-H], 301.2 [ellagic acid-H]. On the basis of the chromatographic properties compound F_3 was suspected to be ellagitannin. Supporting evidence, achieved from the complete acid hydrolysis that gave ellagic acid in the organic phase and glucose in the aqueous phase. Negative ESI-MS spectrum showed the molecular ion peak $[M-H]^-$ at m/z 784, which corresponds to an ellagitannin compound with two moieties attached to glucose sugar while the molecular ion fragment: 482 $[M- HHDP-H]^-$ indicating a loss of HHDP group giving a more confirmation of the presence of bis-HHDP-glucose structure. ^{1}HNMR spectrum: exhibited four singlets signal each integrated to one proton at about δppm 6.6, 6.5, 6.4, 6.3 characterizing a pair of HHDP groups. The down field located anomeric proton at about δppm 5.4 with large *J*-value (9.5 Hz) indicating a β-configuration of glucose moiety. In the structure depending on the characteristic splitting pattern (multiplicity and *J*-values) of the down field located protons signals of glucose moiety in the aliphatic region (δ > 4ppm), the position of esterification were confirmed. The strong downfield shift of H-1 at δ 5.35, H-2 at δ 5.45, H-3 at δ 4.84, H-4 at δ 4.74 indicative the location of the two HHDP groups on OH-1/OH-2 and OH-3/OH-4 of the glucose. Final confirmation was carried out by ^{13}CNMR spectrum that showed the characteristic carbonyl carbon resonance at about δ 168, 166, 165, and 163 for the pair of HHDP groups. All ^{1}H and ^{13}C resonances were assigned by comparison with corresponding published data[31–33]. Thus compound F_3 was identified as 1,2:3,4-bis(s)-hexahydroxy diphenoyl-*β*-D-glucopyranose, which isolated for the first time from genus *Callistemon*.

Compound F$_4$ Off white amorphous powder (22 mg). Chromatographic properties: R_f values; 0.32 (S_1), 0.71 (S_2); dark purple spot under short and long UV-light, it gave deep blue color with $FeCl_3$ reagent and positive response toward specific spray reagents of ellagitannins on PC, gave rose red color turned to dark brown with HNO_2 reagent.[37] On complete acid hydrolysis, it produces ellagic acid in organic phase and glucose in aq. phase (COPC). ^{1}HNMR (500 MHz, DMSO-d6), δppm 6.31, 6.30 (each 1H, s, H-6` α/β), 6.20, 6.19 (each 1H, s, H-6`` α/β), 5.20 (1H, d, *J*=3.8 Hz, H-1α), 5.09 (1H, t, *J*=9.9 Hz, H-3α), 4.83 (1H, t, *J*=9.9 Hz, H-3β), 4.81 (1H, d, J=9 Hz, H-1β), 4.70 (1H, dd, *J*=9.9, 3.8 Hz, H-2α), 4.47 (1H, t, *J*=9.9, H-2β). Negative ESI-MS m/z 481.41$[M-H]^-$, 301.51 [ellagic acid=H]$^-$. Accordingly, compound F_4 was tentatively identified as ellagitannin on the basis of its chromatographic properties and the products of complete acid hydrolysis. On addition, Negative ESI-MS gave a molecular ion peak and fragment ion peak at m/z 481 and 301 which assigned to $[M-H]^-$ and [ellagic acid=H]$^-$. This indicates a hexahydroxydiphenoyl-D-glucose structure.[39] The location of HHDP group on C-2 and C-3 as a biester was indicated from the downfield shift of H-2 and H-3 resonances. The duplication of the sugar protons resonances together with δppm and *J*-values confirmed the sugar moiety as α/β glucopyranose. The presence of one HHDP group was evidenced from the aromatic signals at 6.31, 6.3 and 6.20, 6.19 of H6` and H6`` in both anomers. So according to the previous data and by comparison of the NMR data with the published ones[25,34] and also CoPC with authentic sample, compound F_4 was identified as 2,3-*O*-hexahydroxydiphenoyl-(*a*/*β*)-glucopyranose (Nilocitin) which is isolated for the first time from genus *Callistemon*.

Compound F$_5$ Yellow amorphous powder (40 mg). Chromatographic properties: R_f valus; 0.56 (S_1), 0.65 (S_2); dark purple spot under UV-light turned to dark yellow fluorescence on exposure to ammonia vapors; it gave deep green color with $FeCl_3$ and orange fluorescence with Naturstoff reagent. UV spectral data: λ_{max} (nm) (MeOH): 232,259,354; (+NaOMe): 236,271,405; (+NaOAC): 237,272,320(sh),395; (+NaOAC/ H_3BO_3): 238,260,300 (sh),378; (+$AlCl_3$): 236,268,300 (sh),415; (+$AlCl_3$/ HCl): 235,267,305(sh),400. Complete acid hydrolysis producing glucouronic acid in aq. phase and quercetin in organic phase (COPC). ^{1}HNMR (500 MHz, DMSO-d6): δ ppm 8.14 (1H, d, *J*=2.0 Hz, H-2`), 7.33 (1H, dd, *J*=8.4, 2 Hz, H-6`), 6.79 (1H, d, *J*=8.4 Hz, H-5`), 6.35 (1H, d, *J*=2.0 Hz, H-8), 6.15 (1H, d, *J*=2.0 Hz, H-6), 5.22 (1H, d, *J*=3.4 Hz, H-1``). $^{13}$CNMR (125 MHz, DMSO-d6), δ ppm 178 (C-4), 173.05 (C-6``), 165.32 (C-7), 161.52 (C-5), 157.31 (C-2), 157.08 (C-9), 148.97 (C-4`), 145.33 (C-3`), 134.52 (C-3), 121.21 (C-6`), 121.22 (C-1`), 118.31 (C-5`), 115.97 (C-2`), 103.29 (C-10), 100.03 (C-1``), 94.54 (C-6), 90.06 (C-8), 77.16 (C-3``), 74.64 (C-5``), 72.85 (C-2``), 72.27 (C-4``). Negative ESI-MS Figure 22: m/z 477.32 $[M-H]^-$, 301.51 [quercetin- H]$^-$, 955.16 $[2M-H]^-$. Compound F_5 was expected to be quercetin 3-*O*-glycoside on the basis of its chromatographic properties and UV spectral data. The UV spectra in MeOH showed the two characteristic absorption maxima at λ_{max} 259 (band II) and 354 (band I) for a quercetin aglycone.

The bathochromic shift in band I without decrease in the intensity observed with NaOMe indicated the presence of 4`-OH group. The bathochromic shift in band II observed on the addition of NaOAC indicated a free 7-OH group while the bathochromic shift resulted in band I after addition of H_3BO_3 revealed the presence of an ortho-dihydroxy function in ring B. Moreover the bathochromic shift of band I in $AlCl_3$ together with hypsochromic shift observed after addition HCl confirmed the presence of ortho-dihydroxy B ring, however there is still a bathochromic shift relative to MeOH indicating the presence of a free 5-OH group.[26] On complete acid hydrolysis, F_5 gave glucouronic acid in aq.phase and querectin in organic phase, suggesting quercetin 3-*O*-glucuronide structure. This finding was in accordance with the M.wt. at 478, which was given from molecular ion peak at m/z 477.32 $[M-H]^-$, Moreover the aglycone was identified as quercetin due to the fragment ion peak at 301.51 [quercetin-H]$^-$. ^{1}HNMR spectra, showed an ABX spin coupling system at δ ppm 8.14, 7.33 and 6.79 for H-2`, H-6` and H-5` of 3`,4`-dihydroxy B ring. Additionally the AM coupling system of two meta coupled protons at 6.35 and 6.15 for H-8, H-6, respectively to indicate 5, 7-dihydroxy ring A. The identification evidence of sugar moiety as α-glucuronic acid was in turn reinforced from the presence of α-anomeric protons signal at δ 5.22 (3.4 Hz) for glucuronyl moiety attached at C-3[26]. The structure of the compound F_5 was finally confirmed by the complete assignment of all C-resonances in its ^{13}CNMR spectrum. It showed a typical 15 ^{13}C resonances for 3-*O*-substitiuted quercetin[41]. The sugar moiety was confirmed as glucuronic acid due to resonance of C-6`` at 173.05 ppm and also the rest of carbon resonance at 77.16, 74.64, 72.85 and 72.27 for C-3``, C-5``, C-2`` and C-4``, respectively for the glucuronyl moiety. All ^{1}H and ^{13}C resonances were assigned by comparison with the corresponding published data of structural related compounds[35]. Thus compound F_5 was identified as Querectin 3-*O*-*a*-L-glucuronopyranoside, which is isolated for first time from genus *Callistemon*.

Compounds F_{6A}, F_{6B} Yellow amorphous powder (45 mg). Chromatographic properties: R_f values; 0.43 (S_1), 0.52 (S_2); dark purple spot under UV-light turned to dark yellow fluorescence on exposure to ammonia vapors; it gave deep green color with $FeCl_3$ and orange fluorescence with Naturstoff reagent. UV spectral data: λ_{max} (nm) (MeOH): 256,355; (+NaOMe): 270,408; (+NaOAC): 264,379; (+NaOAC/H_3BO_3): 258,377; (+$AlCl_3$): 270, 300,356,430; (+$AlCl_3$/ HCl): 269,300,370. ^{1}HNMR, ^{13}CNMR, Negative ESI-MS (Table 1). Based on the chromatographic properties and UV- spectral data[26] as described before in F_5, compound F_6 was expected to be quercetin-3-*O*-glycoside. Complete acid hydrolysis of compound F_6 resulted in quercetin in organic phase, glucose and galactose in aqueous phase, suggesting the presence of either quercetin 3-*O*-diglycoside or two compounds which are quercetin 3-*O*-glucoside and quercetin 3-*O*-galactoside. The Negative ESI-MS, showed a molecular ion peak at m/z 463.31 $[M-H]^-$ as the base peak, together with an adduct dimeric ion peak at m/z 927.08 $[2M-H]^-$ which were consistent with the M.wt 464 for either querectin 3-*O*-glucoside or galactoside. This finding was more confirmed with ^{1}HNMR spectrum that showed a duplication of the signals with the same ratio indicating the presence of two compounds. It showed a typical proton signals for querectin aglycone (Table 1)[35] as described before

Table 1. ^{1}H, ^{13}CNMR and Negative ESI/MS data of compounds F_{6A}, F_{6B}.

	F_{6A}		F_{6B}			F_{6A}		F_{6B}	
C. No.	^{13}CNMR	^{1}HNMR	^{13}CNMR	^{1}HNMR	C. No.	^{13}CNMR	^{1}HNMR	^{13}CNMR	^{1}HNMR
2	156.85		156.85		3`	145.22		145.21	
3	133.93		133.76		4`	148.96		148.90	
4	177.94		177.89		5`	116.62	6.8 (9.2)	116.56	6.77 (8.4)
5	161.59		161.6		6`	122.29	7.53 (9.2, 2.3)	122.15	7.52 (8.4, 2.3)
6	99.28	6.16 (2.1Hz)	99.27	6.15 (2.1)	1``	101.34	5.4 (7.5)	102.38	5.31 (7.6)
7	164.64		164.64		2``	71.67		73.57	
8	94.19	6.39 (2.1)	94.19	6.37 (2.1)	3``	76.15		74.53	
9	156.84		156.84		4``	70.23		68.41	
10	104.44		104.37		5``	76.81		77.76	
1`	121.61		121.6		6``	61.28		60.61	
2`	115.76	7.52 (2.3)	115.75	7.51 (2.3)					
$[M-H]^-$	463.31								
[Quercetin-2H]$^-$	300.43								
$[2M-H]^-$	927.08								

^{13}CNMR (125 MHz, DMSO-d6), ^{1}HNMR (500 MHz, DMSO-d6) Values between Parenthesis represent the *J*-value in Hz

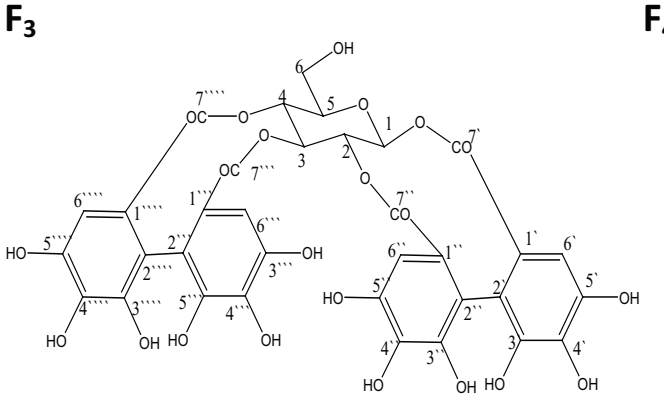
in compound F_5. The sugar moieties were identified as glucoside and galactoside from the presence of two β-anomeric proton signals for glucoside at δ 5.40 (7.5 Hz) and for galactoside at δ 5.31 (7.6 Hz). This evidence was confirmed by ^{13}CNMR exhibited also a duplication of typical 15 ^{13}C- resonance for 3-*O*-substituted quercetin.[41] And the two sugar moieties were proved to be *β*-glucoside and *β*-galactoside from their ^{13}C resonance of 2``, 3``, 4``, 5`` and 6`` and the two *β*-anomeric carbon resonance at 101.34 for glucoside moiety and 102.38 for galactoside moiety. According to the above described data (UV, ESI-MS, NMR) as well as comparing with previous reported data[35–36] and with authentic samples, CoPC, so F_6 was confirmed to be two compounds F_{6A} and F_{6B}. F_{6A} was identified as quercetin 3-*O*-*β*-D-glucopyranoside (Isoquercetin) and F_{6B} was identified as quercetin 3-*O*-*β*-D-galactopyranoside (Hyperin), which is isolated once before from the leaves of *C. lanceolatus*.

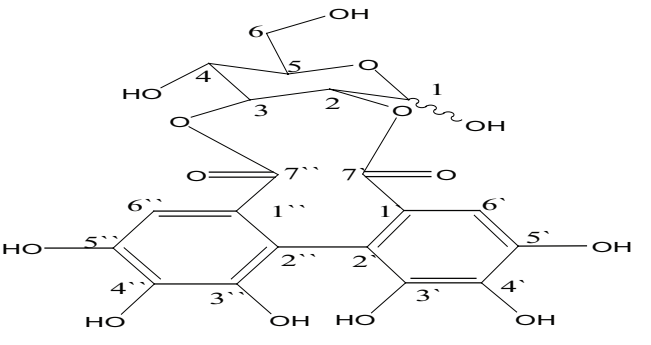
The antimicrobial activity of the alcoholic leaves extract exhibited broad spectrum effect against Gram-positive, Gram-negative and yeasts (Table 2).

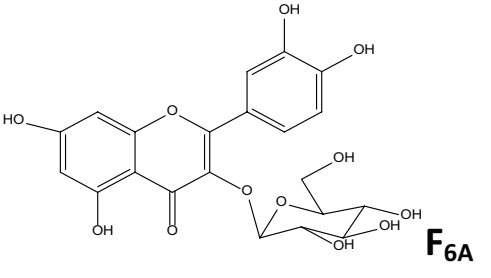
Table 2. Results of antimicrobial activity of the alcoholic extract of leaves of *C. viridiflorus*.

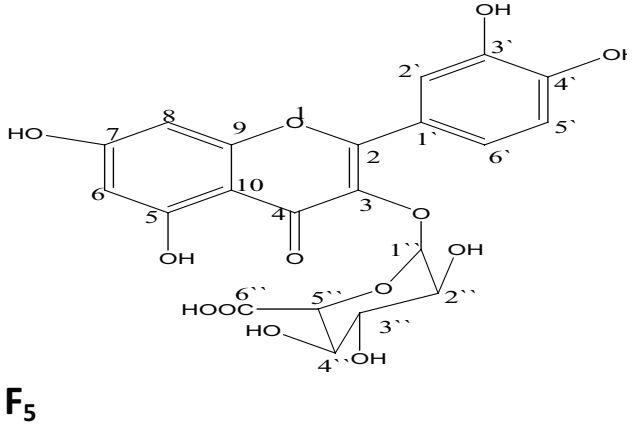
Tested microorganisms	Diameter of zone of inhibition (mm)		
	Extract of leaves	Ofloxacin	Fluconazole
Staphylococcus aureus (gram +ve)	16	30	–
E. coli (gram –ve)	14	26	–
Candida albicans (yeast)	12	–	23

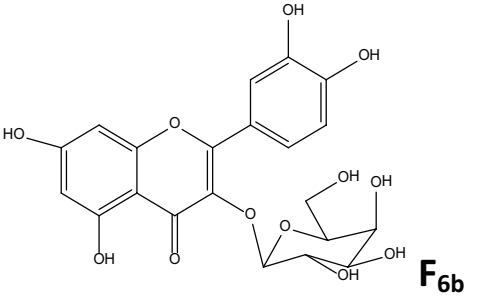
– No inhibition zone.











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Pharmacognostical evaluation of the wild and cultivated variety of Eranda (*Ricinus communis* Linn.) root

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ABSTRACT

Introduction: *Ricinus communis* (Linn), belonging to family Euphorbiaceae, known as Eranda, is used in Ayurvedic system of medicine and its root is recommended for the management of pain, inflammation and infertility, etc. It is available both in wild as well as cultivated conditions. Due to high demand, the cultivated variety is mainly used instead of the other. Hence, to ensure botanical identification and authentication of both varieties harmacognostical evaluation of both the variety was undertaken. **Method:** The present investigation includes macroscopical and microscopical evaluation of both the wild and cultivated root including its powder characteristics following standard procedures. **Result:** Both the roots vary in colour. Roots of both varieties show similar microscopic characters like cork, parenchyma, xylum, phloem and cambium. **Conclusion:** The wild root differs from the cultivated one by being dark greyish-brown and greyish brown in colour, respectively. Both the varieties have same pharmacognostical characters except the presence of tyloses in the wild variety.

Keywords: *Ricinuus communis*, inflammation, infertility, Eranda, root.

INTRODUCTION

Pharmacognosy has become one of the pillars in areas like pharmacy, medicine, natural product chemistry and many others allowing scientists to recognize the importance of plants as sources of medicines. This approach has initiated active research programmes either to isolate new lead compounds or to produce standardized extracts.[1] For this it is very necessary to evaluate various qualitative and quantitative parameters, which may be helpful in setting standards for particular medicinal plant/parts of the plant. With the help of these standards one can easily identify and characterize an individual drug, which may play a major role in maintaining quality and purity of that particular drug and its formulation and prevent it from being adulterated by drug of same or other genius having low potency.[2]

The present study deals with the standardization of one such medicinal plant *Ricinus communis* Linn. (Euphorbiaceae), a soft-wooded small tree widespread throughout the tropics and warm-temperature regions of the world. In the Ayurvedic system of medicine, the leaf, root and seed oil of this plant have been used for the treatment of inflammation and liver disorders.[3] Its root has been highlighted as one of the best drugs having Virshya (androgenic) and vatahara (anlagesic and anti-inflammatory) activities.[4] It is reported that this plant possesses hepatoprotective,[5,6] antidiabetic,[7] laxative[8] and antifertility[9] activities. Methanol extract of root shows anti-inflammatory and free radical scavenging activity.[10]

R. communis is available both in wild as well as cultivated conditions. Due to the high consumption of its root as an Ayurvedic raw drug, the roots of the cultivated variety are utilised mainly instead of the naturally available wild one. In this paper, a study of the transverse section of the root, along with the powder microscopy of both varieties of *R. communis* Linn. was carried out.

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MATERIAL AND METHODS

Collection of drug

Fresh roots of wild (more than six months) and cultivated variety (six months old) were collected after proper identification of the plant as *Ricinus communis* Linn. (Euphorbiaceae), from the adjacent area of Jamnagar town of Gujarat, India, with the help of a taxonomist and a specimen (no. 1491) of the same was preserved in the department, for further reference. The obtained roots were shade dried and made into coarse powder with the help of a mechanical grinder.

Macroscopical evaluation/organoleptic evaluation

Various parameters of the plant material, such as size, shape, colour, odour and taste of the roots were recorded.[11,13]

Microscopic evaluation

Thin free-hand sections of the roots were made and washed with chloral hydrate solution. The stain was made with phloroglucinol and conc. HCl solution. The oil globule was observed by adding sudan III.[12,13] Diagnostic characters in TS and powder of roots of both wild and cultivated varieties were studied with and without staining. Microphotographs were taken using Carl Zeiss binocular microscope.

RESULTS

Macroscopic/organoleptic characters

Wild root: The secondary roots are deeply wavy, dark greyish-brown in colour, 4–12 cm long, and 1–2 cm thick.

Cultivated root: Fresh root are minutely wavy, having secondary roots, greyish-brown to light brown in colour, cut surface brownish-cream, cylindrical, 5–16 cm long, 1–3 cm thick (Figure 1).

Microscopic characters

Transverse section of wild fresh root

Diagrammatic section: The diagrammatic sketch shows outer cork, reduced cortex and wide stealer region composed of compactly arranged xylem vessels with xylem parenchyma and fibers separated by uniserriate to biserriate medullary rays.

Cork: Mature root shows 4–5 or more layers of tangentially elongated, radially arranged cork cells.

Cortex: Composed of oval to polygonal, parenchymatous cells; in which starch grains, rosette crystals, schlerenchymatous cells are observed.

Phloem: Composed of sieve tubes, parenchyma, fibers and groups of fibers traversed by 2–8 cells wide medullary rays.

Cambium: Consists of 2–4 layer of rectangular shaped cells situated just under the phloem.

Xylem: Consists of usual elements, xylem vessels of various sizes, occur in singles and groups of 2–5 cells arranged radially having reticulate thickening; xylem medullary rays 2–4 cells wide, tyloses are observed.

Transverse section of cultivated root

Diagrammatic section: The transverse section of the cultivated root shows outer cork, reduced cortex and wide stealer region composed of compactly arranged xylem vessels with xylem parenchyma and fibers separated by uniserriate to biserriate medullary rays.

Cork: Root of the cultivated variety shows 3–4 layers of cork which is tangentially elongated and it is arranged in radial form.

Cortex: It is composed of parecnchymatous cells which are polygonal in shape, in which starch grains, rosette crystals, stone cells are observed.

Phloem: Composed of sieve tubes, parenchyamatous cells, fibres and layers of medullary layers are present transversally.

Root of wild variety | Root of cultivated variety | Powder of wild variety | Powder of cultivated variety

Figure 1. Morphology of Eranda root wild and cultivated.

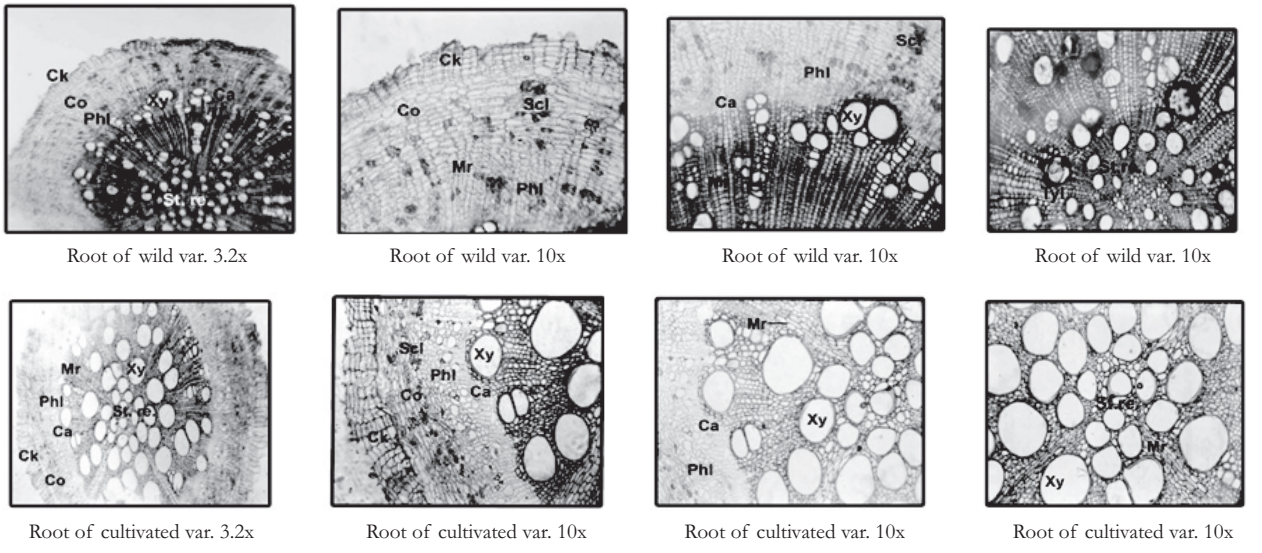

Figure 2. Microphotograph of transverse section of Eranda root wild and cultivated.
Ck – Cork, Co – Cortex, Phl – Phloem, Xy – Xylem, St.re – Stele region, Mr – Medulary Rays, Ca – Cambium, Scl- Sclerenchyma, Ty – Tyloses.

Cambium: Consists of 2–3 layers of rectangular shaped cells situated under the phloem.

Xylem: It consists of xylem vessels of pentarch to hexarch, oil globules, medullary rays 2–3 cells wide with plenty of starch grains in it (Figure 2).

DRIED POWDER MICROSCOPIC CHARACTERS

Wild root

Brownish-cream to darkish in colour, The diagnostic characters are cork in surface view from cork, starch grains, rosette crystals from cortex zone, border pitted vessel and fibers from vascular bundles, yellow colour content are seen from cortex zone, prismatic crystals are from cortical region (Figure 3).

Cultivated root

Powder microscopy of cultivated variety shows plenty of starch grains and rosette crystals from cortical region, pitted vessels from vascular bundles, cork cells in surface as well as in tangential view from cork, prismatic crystals from cortex, fibers from phloem (Figure 4).

DISCUSSION

Each and every species has its own pharmacognostical features, which determine the authenticity of the particular

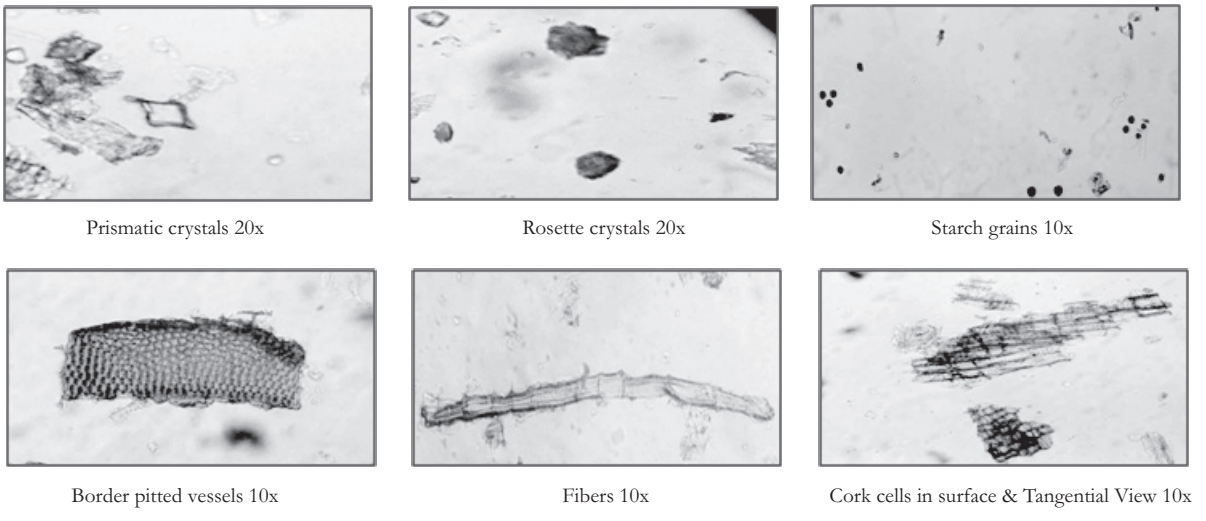

Figure 3. Microphotograph-wild variety of Eranda root powder.

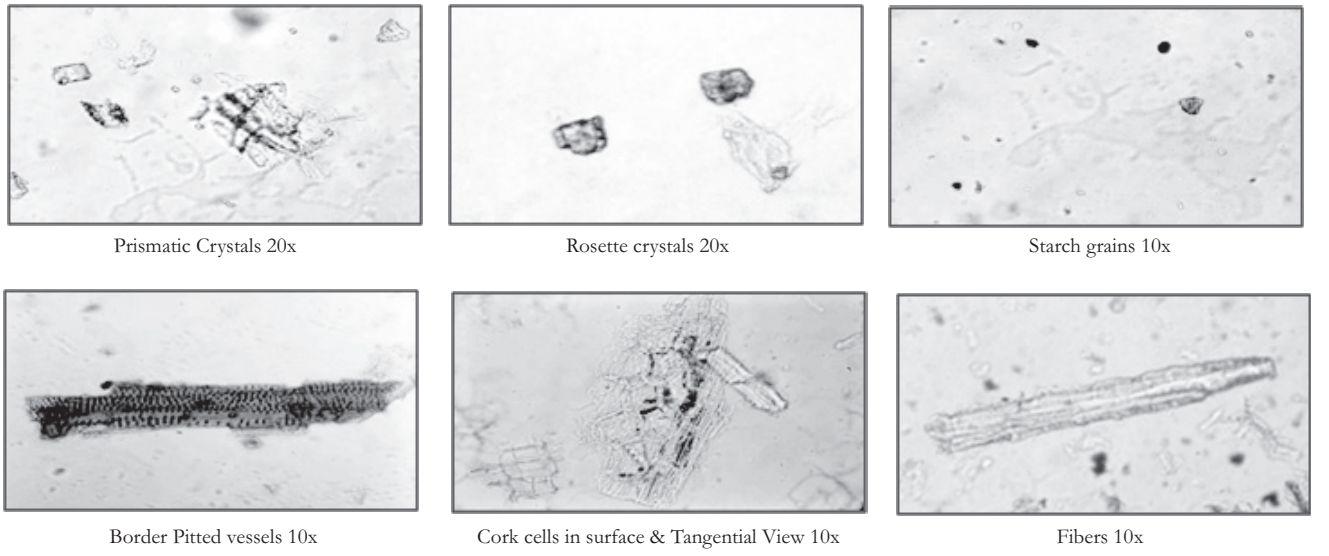

Figure 4. Microphotograph-cultivated variety of Eranda root powder.

Table 1. Pharmacognostical comparison of wild and cultivated varieties of *R. Communis* root.

	Characteristic	Wild variety	Cultivated variety
Macroscopical characteristic	Color	Dark grayish brown	Grayish brown
	Odor	Odorless	Odorless
	Texture	Rough	Rough
	Taste	Tasteless	Tasteless
Microscopical characteristic	Cork	+	+
	Parenchyma	+	+
	Phloem	+	+
	Cambium	+	+
	Xylem	+	+
	Tyloses	+	-
Powder characteristic	Prismatic crystals	+	+
	Rosette crystals	+	+
	Starch grains	+	+
	Border pitted Vessels	+	+
	Cork cells	+	+
	Fibers	+	+

drug. It helps in the identification and determination of the quality and purity of the raw drug. The microscopical study reveals that the most of the characters are the same in the wild and cultivated varieties of Eranda root (like cork, cortex, cambium, xylem, and phloem). The powder microscopy shows that the characters like cork cells, prismatic crystals, rosette crystals, starch grains, border pitted vessels and fibers are the same in both varieties Eranda root (Table 1).

CONCLUSION

The wild roots of *R. communis* are more wavier and also darker in colour than the cultivated one. Both the varieties have the same pharmacognostical characters except the presence of tyloses in the wild variety. However, this inference should be made along with phytochemical, pharmacological and clinical trials.

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Isolation and identification of antibacterial compound from diethyl ether extract of *Plantago major* L

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ABSTRACT

Plantago major L. is a plant of the Plantaginaceae family which has been investigated on diethyl ether extract generate active spot as an antibacterial. The aim of the investigation was to identify the antibacterial compound from diethyl ether extract of daun sendok. as much 500 gram material was extracted maceration method was obtained 50 gram methanol extract, continued by partition with diethyl ether obtained 19.5 gram dried extract, the extract diethyl ether which isolated by suction column chromatography obtained 3 fractions (F1, F2 and F3). The third fraction (F3) was isolated by column chromatography obtained 2 fractions (3-1 and 3-2). The 3-1 active fraction (inhibit the *Escherichia coli*) isolated by preparative-TLC obtained 2 compounds. Compound 1 was active, single spot and visualization with Lieberman-Buchard and vanillin-sulfate acid. It have a steroid compound.

Keywords: *Plantago major* L., antibacterial, diethyl ether extract.

INTRODUCTION

Plantago major L. is widely spread in Asia and the Mediterranean countries; the plant is cultivated extensively in India and Pakistan and adapts to western Europe and subtropical regions[1,2]. Usually wrinkled and contracted leaf and spike, grayish green to dark yellowgreen in colour; when soaked in water and smoothed out, the lamina is ovate or orbicular-ovate, 4–15 cm in length, 3–8 cm in width; apex acute, and base sharply narrowed; margin slightly wavy, with distinct parallel veins; glabrous or nearly glabrous; petiole is rather longer than the lamina, and its base is slightly expanded with thin-walled leaf-sheath; scape is 10–50 cm in length, one-third to one-half of the upper part forming the spike, with dense florets; the lower part of inflorescence often shows pyxidia; roots usually removed, but, if any, fine roots are closely packed[2]. The antidiarrhoeal effects of Semen Plantaginis have been extensively investigated in patients with acute and chronic diarrhoea. An increase in the viscosity of the intestinal contents due to the binding of fluid and an increased colonic transit time (decreased frequency of defecation) were observed in patients treated with the drug.[3,4] The aim of the investigation was identify antibacterial compound from diethyl ether extract of *Plantago major* L.

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MATERIALS AND METHOD

Collection of plant material

Fresh plant parts collected from Toli-Toli regency, South East Celebes, the taxonomic identities are preserved at Pharmacognosy Laboratory, Faculty of Pharmacy, Moslem University of Indonesia.

Extraction and isolation of plant materials

Dried leaf (0.5 kg) was macerated with 0.5 liter methanol in five days (this process was done six times), then evaporated which gave 30 gm gummy extract. The gummy extract was dissolved in 80 ml diethyl ether, the diethyl ether fraction was isolated by suction column. The active fraction was continued for isolation using column chromatography method, the column was parked with silica gel G. 60 (Merck) and 0.6 g of the extract was placed on top of the silica and eluted with chloroform:ethyl acetate (1:1), (1:50), (1:10) elution in each eluent end. If the solution of fraction

was clear then it was replaced with the other eluent until the last variation. Each fraction from variation of eluent was collected and evaporated at the room temperature.

MICROORGANISMS

The following test organisms were used: *Pseudomonas aureginosa, Staphylococcus epidermidis*, *Vibrio sp.* and *Escherichia coli.*

Screening for antibacterial activities

The 10 mg of extracts were dissolved in 0.2 ml dimethylsulfoxide (DMSO) and added with 9.8 of GNA medium to a final concentration of 1 mg/mL, bacteria were cultured at 30° C for 24 h.

Antibacterial activity was determined by TLC bioautography method.

TLC bioautography

The methanol extract was used for TLC bioautography. Plates were developed with chloroform:ethyl acetate (10:1), which separated components into wide range of Rf values. The components were visualized under visible light. Developed TLC plates were carefully dried for complete removal of the solvents and overlaid by nutrient agar seeded with an overnight culture of the bacteria test. The plate was incubated for 24 h at 37° C and then sprayed with Dragendorf and Liebermann-Burchard reagent. Inhibition zones were observed as clear spots against purple background and their Rf values were compared with the reference plate.[5]

RESULTS AND DISCUSSION

Result of extraction is shown in Table 1.

The *in vitro* antibacterial activity of diethyl ether extracts and isolate of dried *Plantago major* L. shows that it has activity to inhibit the *Escherichia coli*, as shown in Table 2.

Table 1. Result of Isolation 0.5 Kg *Plantago major* L. using masceration method.

No	Sample	Weight (g)
1	Fresh sample	4.905
2	Dried sample	
3	Methanol extract	50
4	Diethyl ether extract	19.7

Table 2. Inhibitory Activity of diethyl ether extracts and isolate of *Plantago major* L.

Bacteria	Activity	
	Extract	Isolate
Pseudomonas aureginosa	–	–
Staphylococcus epidermidis	–	–
Vibrio sp	+	–
Escherichia coli	+	+

Note : (-) = No Inhibition; (+) = Inhition

The diethyl ether extract fractionated by suction column give three fractions. The active fraction continued for isolation using column chromatography method is shown in Table 3.

The diethyl ether extract of *Plantago major* L. displayed activity on *E. coli*. This is suitable with the results of (Çitoğlu & Altanlar, 2003) who reported that the aqueous ethanolic extracts of *Plantago major* L. when compared with standard antibiotics, were found to have good activity against *E. coli* extract showing a stronger and broader spectrum of antibacterial activity.[6] This research used suction column for fractionating the extracts to decrease time for isolation and trough microbial assay to know where the active compound is present and possible to be continued in to the next isolation with column chromatography method.[7]

Results of the antibacterial assay showed that the extract and isolate are only active on the Gram-positive bacteria (*E. coli* and *Vibrio sp.*). The reason for the different sensitivity between Gram-positive and Gram-negative bacteria could be ascribed to the morphological differences between these microorganisms.

Table 3. Rf value and spot color of the third fraction (*Plantago major* L.) using suction colomn method.

Spot	Rf			Color		
	UV 254	UV 366	H_2SO_4	UV 254	UV 366	H_2SO_4
1	0.23	0.23	0.23	Green	Blue	Brown
2	0.19	0.19	0.19	Orange	Violet	Green
3	0.14		0.14	Green		Yellow

Note :
TLC Plate = Silica gel G. 60 F_{254}
Eluent = n-hexsane : ethyl acetate (3:1)
Active spot = 2

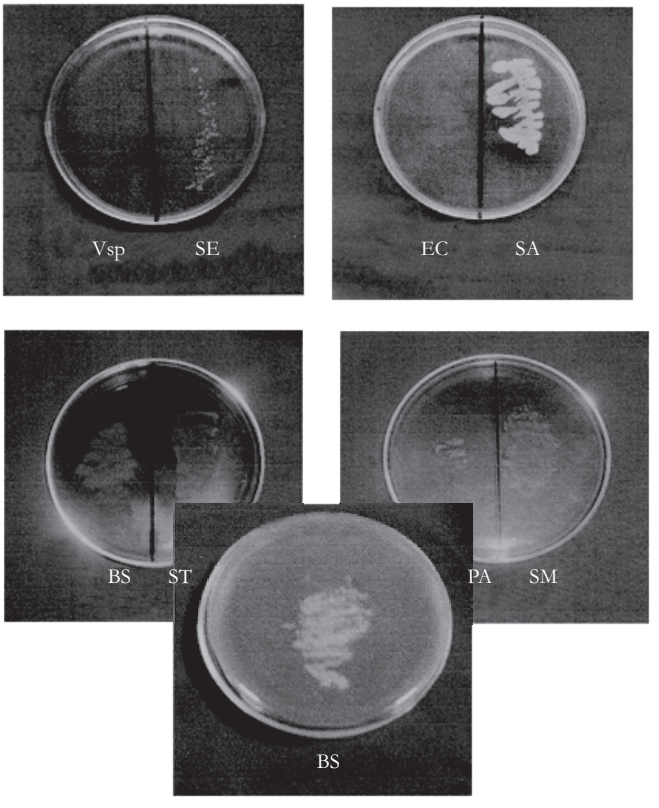
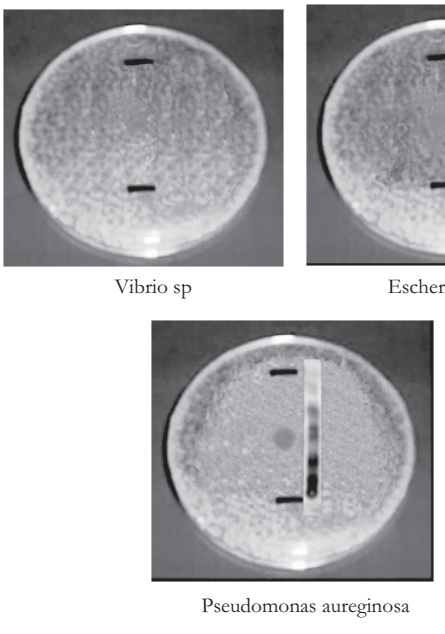


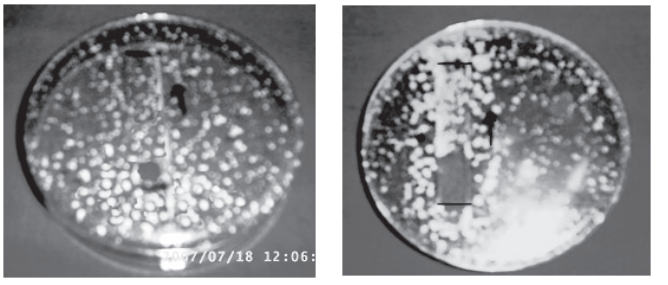
Figure 1. Result of antimicrobial screening from diethyl-ether extract of *Plantago mayor* L. leaf.
Note : Vsp = *Vibrio* sp, ST = *Salmonella thyposa*
SE = Staphylococcus epidermidis, PA = Pseudomonas aeruginosa
EC = Escherichia coli, SM = Streptococcus mutans
SA = Staphylococcus aureus, CA = Candida albicans
BS = Bacillus subtilis



Vibrio sp | Escherichia coli

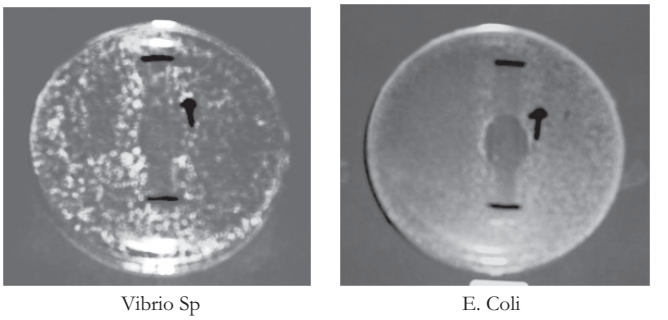
Pseudomonas aureginosa

Figure 2. Result of TLC-Bioautography from diethyl ether extract of *Plantago mayor* L.



Vibrio Sp | E. Coli

Figure 3. Result of TLC-Bioautography from third fraction of *Plantago mayor* L.



Vibrio Sp | E. Coli

Figure 4. Result of TLC-Bioautography from Isolate of *Plantago mayor* L.

Gram-negative bacteria have an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da.[8]

The Rf values and colour band of the third fraction has been summarized in Table 3. TLC bioautographic assay allowed outlining the chemical profile of the third fraction thus detecting the active substances that presented antimicrobial activity. The results of this assay revealed that compounds eluted at Rf 0.19, exhibited strong antibacterial activity against *E. coli* therefore, suggesting the presence of antimicrobial active substances. The phytochemistry assay on the active spot with Lieberman-Buchard reagent and vanillin-sulfate acid showed that the active compound is steroid.

CONCLUSION

Based on these results, it is possible to conclude that *Plantago major* L. has a strong antibacterial activity against bacteria and the extract can be used as an antibacterial agent for diarrheal effects of *E. coli*.

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Effect of NaCl salinity on the growth and mineral nutrition of one month old *Prosopis juliflora* (Sw.) DC seedlings

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ABSTRACT

The effect of NaCl salinity on the growth and mineral nutrition of one month old *Prosopis juliflora* seedlings was studied with the help of sand culture experiment. The seedlings were treated with 100, 200 and 300 mM NaCl concentrations, respectively. It was found that there was stimulation in plant growth at low concentrations of salt, while at higher concentrations it was hampered causing a marked decrease in the fresh (60%) and dry weight (80%). The level of sodium, chloride and calcium was found to increase in the roots as well as leaves with increasing levels of salinity in the rooting medium. The level of potassium and phosphorus however, was found to decrease in the leaves and roots of seedlings grown under NaCl stress.

Keywords: salinity, growth, minerals, *Prosopis juliflora*.

INTRODUCTION

Salinity is a major environmental obstacle in crop growing areas, which limits seed germination and seedling growth.[1] Plantation in saline soil requires selection of salt-tolerant species as germination is critical in such soils.[2] According to recent observations, about 6.73 million ha land in India is saline.[3]

Prosopis juliflora is a multipurpose plant of great value. It provides timber, firewood and live stock feed. The capacity of biomass production in *Prosopis* is high and it has good ability to grow in the poorest type of soil, under very arid conditions where few other species survive.[4] According to Basavraja et al.,[5] it is an efficient tree species for reclamation of salt-affected soils. The workers from Central Soil Salinity Research Institute (CSSRI) have also noticed that genus *Prosopis* can be used for afforestation of salt-affected land. It is observed that *Prosopis juliflora* can be successfully established in a farmland of a village (M-Digraj, Dist-Sangli, Maharashtra, India), heavily affected by secondary salinisation. As Na and Cl are the dominant salts in a salt-affected area, an attempt has been made to study the effect of NaCl salt on the growth and mineral nutrition of *Prosopis juliflora* seedlings grown under salt stress in laboratory conditions.

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MATERIALS AND METHODS

Mechanically scarified seeds were used to raise the seedlings in sand culture. Seeds were obtained from *Prosopis juliflora* plants growing in the salt-affected agriculture fields in the months of April-May. After the germination of seedlings (10 days old), they were treated with increasing concentrations of salt (100, 200 and 300 mM NaCl) mixed with half strength Hoagland solution. The treatments were given twice a week alternating with watering the plants. Forty-day old seedlings were then harvested and measurements were taken for fresh weight by a digital balance. Dry weight was determined after drying the seedlings in oven at 40°C for 8 days. Acid digestion method of Toth et al.[6] has been followed for the analysis of inorganic constituents. Sodium, potassium and calcium were estimated using atomic absorption Spectrophotometer. The method of Sekine et al.[7] was employed for estimation of phosphorus from the roots and leaves. Extraction of chloride was done according to the method described by Imamul Huq and

Larher[8] with slight modifications and estimated according to the method of Chapman and Pratt.[9]

RESULTS AND DISCUSSION

The effect of NaCl salinity on average fresh weight of 40-day old *Prosopis* seedlings is shown in Figure 1. It is observed that salinity treatment has caused a marked decrease in fresh weight of a seedling at higher concentrations of salt but at lower concentrations there was an increase in the fresh weight. The fresh weight of a plant is an important parameter for the assessment of growth since it represents the total fresh biomass. In glycophytes, reduction in biomass is directly co-related with the rate of stress applied (Storey and Wyn Jones).[10] Karadge and Chavan[11] stated that low salt level increases biomass in *Sesbania*-restricted Na uptake but it decreases with increasing salinity. Fresh weight of Celery shoot was enhanced by low NaCl level (Everad et al.).[12] Venketsen et al.[13] observed an increase in the fresh weight of *Ipomea* plants up to 200 mM NaCl. Viegas et al.[14] also noticed that growth was stimulated at low NaCl levels in *Prosopis juliflora*.

In *Prosopis juliflora* also, an increase in the biomass in plants subjected to 100 mM NaCl is noticed. However, at very high concentrations (300 mM NaCl) about 60.44% decline in fresh weight is evident. But still at this concentration, the plants survive. Since *Prosopis juliflora* is a perennial tree species and the present analysis is performed at 1-month growth stage, it is quite probable that after initial hardening, the plant may become highly salt tolerant at later stages of growth and show better biomass production.

Influence of NaCl salinity on dry matter production in *Prosopis juliflora* is depicted in Figure 1. It is evident from the figure that dry weight increased in plants subjected to 100 mM NaCl while at higher concentrations it decreased. The dry weight of stem tissue also shows a similar pattern.

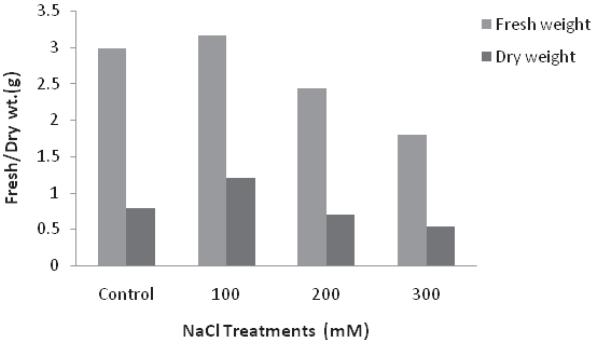

Figure 1. Effect of sodium chloride salinity on fresh and dry weights of *Prosopis juliflora* (Sw.) plants.

In contrast to shoot tissue, the dry weight of the root is higher than control in plants subjected to 100 and 200 mM NaCl and a decline is noticed at 300 mM NaCl concentration. Dry matter production in a plant represents a balance between total photosynthesis and respiration. Salinity is reported to inhibit photosynthetic CO_2 fixation in many plants (Bruria and Feigin).[15] An increase in the rate of respiration and photorespiration in salt-stressed plants is also reported (Downton).[16] Both these factors contribute to decline in dry matter production in salt-stressed plants.

According to Karadge and Chavan[11] the dry matter production in *Sesbania* was affected only at the higher salt concentration. Bottacin et al.[17] have reported that the dry matter production was stimulated up to 150 mM NaCl concentration only in *Pennisetum americanum*. Pessarakli et al.[18] reported that in saltgrass grown under salt stress, the dry weight at 200 and 400 mM NaCl was more than that of control plant. In case of *Prosopis juliflora* the dry matter production is reduced by 68.41% in 300 mM NaCl treated plants, while at 100 mM dose it is stimulated. The dry weight of root tissue is maintained above control in plants treated with 200 mM NaCl and this is certainly an important feature which may contribute to salinity tolerance of this species.

The influence of NaCl salinity on sodium and chloride contents of the leaves and roots of *Prosopis juliflora* is depicted in Figure 2a, b. From the figure, it is clear that due to NaCl treatment the level of sodium and chloride in both leaves and roots is increased. It is evident that the chloride content is increased to a greater extent than sodium.

Accumulation of sodium in plants growing in saline habitat is reported by a number of workers. Ramoliya et al.[19] have studied the mineral uptake by *Prosopis cineraria* plants exposed to salt stress and noticed that the seedlings showed greater accumulation of sodium in the leaf tissue as compared to that in root tissue. Since *Prosopis juliflora* belongs to a category of xerohalophytes (Aronson),[20] besides playing a positive role in metabolism, sodium might also be contributing to osmotic adjustment under saline conditions. NaCl salinity is related with increased contents of Na^+ and Cl^- and to a decreased availability of nutrients arising from lower uptake and the competition between nutrients such as K^+ and NO_3^- with Na^+ and Cl^-, respectively (Bottacin et al.).[17]

The chloride concentration in the root and leaves of *Prosopis juliflora* is found to increase with an increasing level of salinity. The accumulation of this nutrient is more pronounced in the leaf tissue than in the root tissue. Thus along with sodium, is possible chloride is also contributing to osmotic adjustment in this xerohalophyte species.

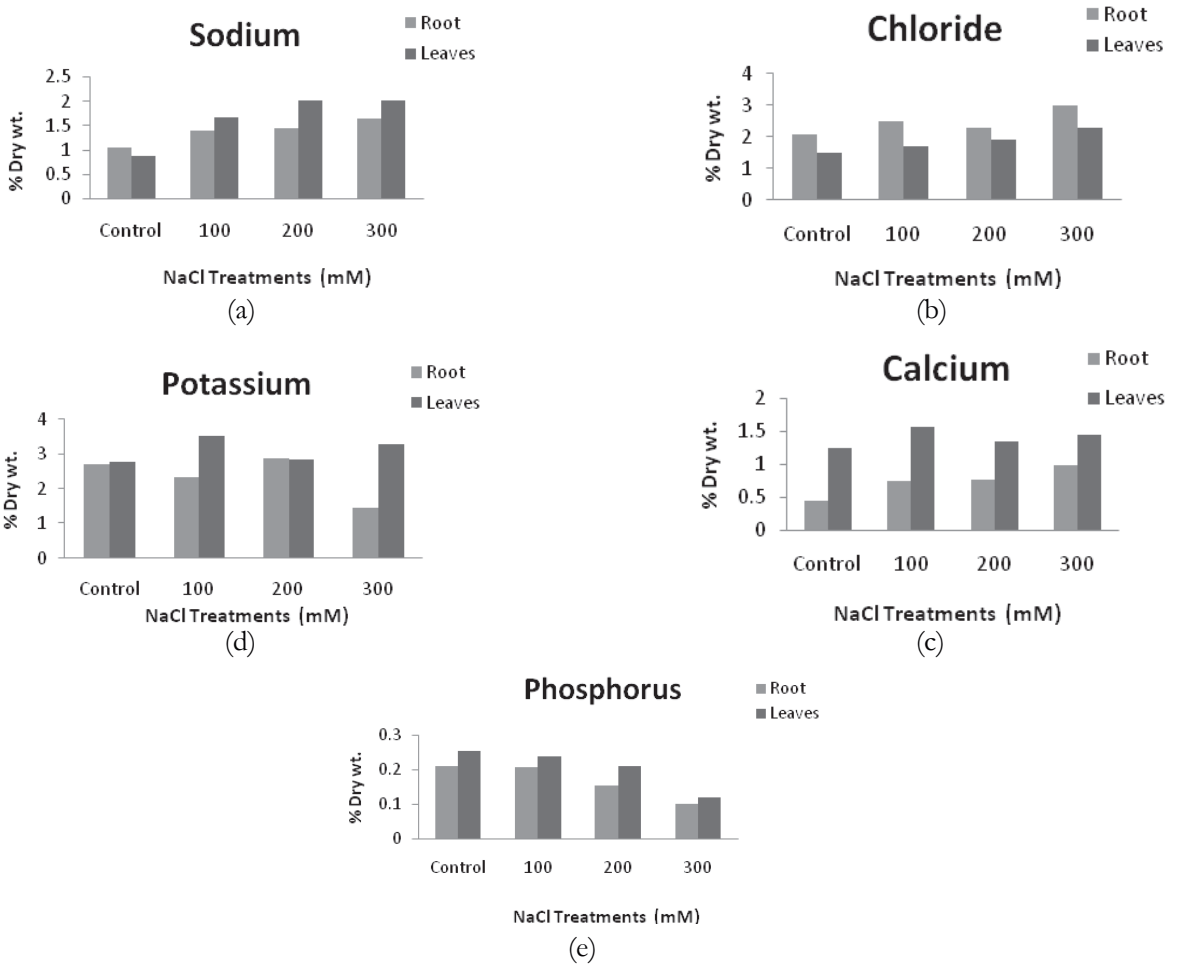

Figure 2. Effect of sodium chloride salinity on (a) Sodium (b) Chloride (c) Potassium (d) Calcium and (e) Phosphorus contents of the roots and leaves of *Prosopis juliflora* (Sw.) plants.

Potassium plays an important role in activation of about 60 plant enzymes, protein synthesis, stomatal movement, photosynthesis and osmoregulation.[21] According to Garbquriioo and Dupont,[22] plants growing in saline soil must maintain a much higher K/Na ratio in their cytoplasm than that present in the surrounding. Since sodium is a dominant monovalent cation, it competes with potassium for common uptake.[11] Reported a decrease in the K content of the roots of salt-stressed *Sesbania aculeta*. A preferential potassium uptake mechanism has been considered as one of the important mechanisms of salt tolerance in marine algae and mangroves.[23] In the present investigation it is noticed that *Prosopis juliflora* plants are able to maintain a potassium level above the optimal level (1%) in both leaves and roots over the entire range of the salt treatment (Figure 2c). The leaf potassium appears to contribute to salinity tolerance in this plant.

Effect of NaCl salinity on the calcium content of the leaves and roots of *Prosopis juliflora* is depicted in Figure 2d. There are many reports of increasing Ca^{++} level in some plant species under saline conditions.[24][25][11] According to Morabito et al.[26] Ca is protective in function and maintains membrane integrity and avoids K leakage under salt stress. Ramoliya et al.[19] observed that calcium content increased in the leaves of *Prosopis cineraria* seedlings subjected to salt stress. In case of *Prosopis juliflora* also calcium might be playing a similar role since its nutrition is not at all affected by salt stress and its level in both leaves and root is elevated in salt stressed plants.

There are many reports indicating the suppression of P uptake due to salt stress.[27,28,29,30] In case of *Prosopis cineraria* seedlings, Ramoliya et al.[19] noticed that phosphorus content significantly decreased in the leaves with an increase in soil salinity while it gradually decreased in stem and root tissues. *Prosopis juliflora* seedlings have shown a pattern (Figure 2e) similar to that in *P. cineraria* since in both root and leaves a decline

in P content was evident in the seedlings exposed to salt stress. A decrease in P content in the leaves and roots of *Prosopis juliflora* seedlings during NaCl stress was also recorded by Patil and Chavan.[31] In contrast to calcium and potassium nutrition, this appears to be quite stable during salt stress in this species. The phosphorus nutrition in *Prosopis juliflora* seems to be sensitive to salt stress. The disturbance in P nutrition can have significant effects on overall plant metabolism in view.

CONCLUSION

It is concluded that due to salinity, there are some changes in the growth and mineral uptake of the salt tolerant *Prosopis juliflora* which are probably related to mechanism underlying salt tolerance in the species.

ACKNOWLEDGEMENT

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Antibacterial activity of *Eachinacia angustfolia*

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ABSTRACT

Antibacterial activity of petroleum ether, methanol and aqueous extracts of *Eachinacia angustfiloa* was investigated. The extracts were tested against both Gram-positive, Gram-negative and fungi organisms *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Staphylococcus epidermidis*, *Aspergillus niger* and *Candida albicans* at different doses of 0.5 mg/ml, 1.5 mg/ml and 1.5 mg/ml using cup plate and minimum inhibitory concentration (MIC) method. The *Eachinacia angustfiloa* extract exhibited broad-spectrum antibacterial activity against the tested organisms. The concentration of the extract that inhibits growth of the organism at a 530 nm MIC was found to be 400, 500, 600 and 900 µg/ml and MBC value was found to be 700, 900 and 1000 µg/ml, respectively.

Keywords: *Eachinacia angustfiloa*, cup plate, MIC, MBC.

INTRODUCTION

Echinacea angustifolia (EEA) is also known as Black Sampson. Echinacea plants are herbaceous perennials reaching 10–60 cm in height. The stem ascends either from a vertical taproot of Compositae family, that is known to have great medicinal importance widely used for the prevention and treatment of acute respiratory infections. It is also known for immune stimulation, anti-cancer and wound healing activity.[1] The plant also reported immunomodulator,[2] anti-inflammatory,[3] anti-stress,[4] anti-cancer,[5] anti-oxidant[6] and wound healing activity.[7] This study is designed to evaluate the antibacterial activity of *Echinacea angustifolia*.

MATERIALS AND METHOD

The ethanolic extract of *Echinacea angustifolia* (ECAG/JA 0071), was a gift sample obtained from Madhur Pharma, Bangalore Hundred grams of dried ethanolic extract of *Echinacea angustifolia* was successively fractioned with various solvents such as petroleum ether (60–80°C), methanol and water having different polarity in separating funnels. After drying, the different extracts were used for antibacterial screening.

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Microorganisms

The microorganisms employed in the current study were procured from the Deccan Medical College, Hydrabad (AP) and Osmania University, Hydrabad (AP) India.

Media

Nutrient broth, nutrient agar, malt extract broth and sabouraud dextrose agar, all products of Himedia Laboratories Mumbai (India) were used in this study.

Antimicrobial agents

Ampicillin, and fluconazole, (1% w/v) were used. The test solutions of the extract were prepared in DMSO at a concentration of 0.5–1.5 mg/ml. Fluconazole was used as standard and was dissolved in sterilized water to get a concentration of 100 µg/0.1 ml The test solutions of the extract were prepared in DMSO at a concentration of 0.5–1.5 mg/ml. Ampicillin was used as standard and was dissolved in sterilized water to get a concentration of 100 µg/0.1 ml DMSO (0.1 ml) was used as solvent control.

Anti-bacterial screening by cup plate method

A sterile borer was used to prepare cups of 10 mm diameter in the agar media spread with the microorganisms.

0.1 ml of inoculums (of 10^4 to 10^6 CFU/ml population prepared from standardized culture, adjusted with peptone water) was spread on an agar plate by spread plate technique. Accurately measured (0.1 ml) solution of each sample and standard were added to the cups with a micropipette. All the plates were kept in a refrigerator at 2–8°C for a period of two hours for effective diffusion of test compounds and standards. Later, they were incubated at 37°C for 24 h. The presence of definite zones of inhibition around the cup indicated antibacterial activity.[8,9,10]

Determination of minimum inhibitory concentration

Two ml of nutrient broth for bacteria and saboraud dextrose broth for fungi was sterilized by autoclaving at 121°C for 15 minutes in different test tubes. 0.1 ml of required suspension (1×10^6 cfu/ml) of microorganism was added. One ml of different dilutions of extracts ranging from 100–1000 µg was added to the inoculated medium. Two ml of sterile nutrient broth inoculated with 0.1 ml of organism was taken as positive control and 2 ml of uninoculated tube of medium is incubated to serve as negative growth control. All the test tubes were incubated at 37 ± 1°C for 24 hr. The lowest concentration of the extract that inhibits growth of the organism, as detected by lack of visual turbidity (matching the negative growth control), is designated as minimum inhibitory concentration and reported as µg of substance per ml.[11,12,13]

RESULTS AND DISCUSSION

The results of antimicrobial activity test of *Echinacea angustifolia* extracts by different solvents with increasing polarity such as water, methanol, petroleum ether are shown in Tables 1, 2 and 3. The different extracts of this solvent vary in their antibacterial activity against the tested bacteria. The aqueous extraction of *Echinacea angustifolia* gave less antibacterial activities than all other organic solvents extraction. The most active organic extract was the methanol and petroleum ether extract. A methanol extraction from *Echinacea angustifolia* produced an inhibition zone of almost 7–18 mm. Minimum Inhibitory Concentration (MIC) value was found to be 900 µg/ml and Minimum bactericidal concentration MBC value was found to be 1000 µg/ml. The aqueous extract displayed no

Table 1. Antibacterial activity of *Echinacea angustifolia* petroleum ether extract.

Organisms	Zone of inhibition (mm)				
	EEA 0.5 mg/ml	EEA 1 mg/ml	EEA 1.5 mg/ml	Ampicillin 100 µg/ml	Fluconazole 100 µg/ml
Staphylococcus aureus (2079)	8	12	18	21	–
Bacillus subtilis (NCIM-2708)	7	13	18	24	–
Staphylococcus epidermidis (2478)	9	11	15	22	–
Escherichia coli (2685)	8	14	16	21	–
Candida albicans (MTCC1344)	7	14	16	–	21
Aspergillus niger (MTCC184)	7	12	13	–	20

Table 1a. Minimum inhibitory concentration, minimum bactericidal concentration, minimum fungicidal concentration of EEA.

Organisms	Concentration in g/ml of EEA		
	MIC	MBC	MFC
Staphylococcus aureus (2079)	500	700	–
Bacillus subtilis (NCIM-2708)	400	600	–
Staphylococcus epidermidis (2478)	400	500	–
Escherichia coli (2685)	500	700	–
Candida albicans (MTCC1344)	800	–	1000
Aspergillus niger (MTCC184)	900	–	1000

Table 2. Antibacterial activity of *Echinacea angustifolia* methanol extract.

Organisms	Zone of inhibition (mm)				
	EEA 0.5 mg/ml	EEA 1 mg/ml	EEA 1.5 mg/ml	Ampicillin 100 µg/ml	Fluconazole 100 µg/ml
Staphylococcus aureus (2079)	6	11	16	21	–
Bacillus subtilis (NCIM-2708)	5	10	13	21	–
Staphylococcus epidermidis (2478)	6	11	14	20	–
Escherichia coli (2685)	8	11	16	22	–
Candida albicans (MTCC1344)	7	13	16	–	21
Aspergillus niger (MTCC184)	4	11	14	–	20

Table 2a. Minimum inhibitory concentration, minimum bactericidal concentration, minimum fungicidal concentration of EREAS.

Organisms	Concentration in g/ml of EEA		
	MIC	MBC	MFC
Staphylococcus aureus (2079)	400	500	–
Bacillus subtilis (NCIM-2708)	300	500	–
Staphylococcus epidermidis (2478)	400	500	–
Escherichia coli (2685)	500	600	–
Candida albicans (MTCC1344)	600	–	900
Aspergillus niger (MTCC184)	700	–	800

Table 3. Antibacterial activity of *Echinacea angustifolia* aqueous extract.

Organisms	Zone of inhibition (mm)				
	EEA 0.5 mg/ml	EEA 1 mg/ml	EEA 1.5 mg/ml	Ampicillin 100 µg/ml	Fluconazole 100 µg/ml
Staphylococcus aureus (2079)	3	6	10	21	–
Bacillus subtilis (NCIM-2708)	4	6	9	24	–
Staphylococcus epidermidis (2478)	3	7	10	22	–
Escherichia coli (2685)	3	6	8	21	–
Candida albicans (MTCC1344)	2	8	11	–	20
Aspergillus niger (MTCC184)	2	6	10	–	18

Table 3a. Minimum inhibitory concentration, minimum bactericidal concentration, minimum fungicidal concentration of EREAS.

Organisms	Concentration in g/ml of EEA		
	MIC	MBC	MFC
Staphylococcus aureus (2079)	300	400	–
Bacillus subtilis (NCIM-2708)	300	500	–
Staphylococcus epidermidis (2478)	200	500	–
Escherichia coli (2685)	200	700	–
Candida albicans (MTCC1344)	400	–	800
Aspergillus niger (MTCC184)	300	–	700

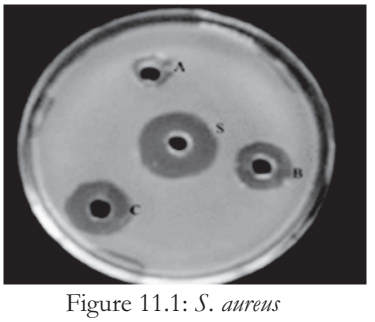

Figure 11.1: *S. aureus*

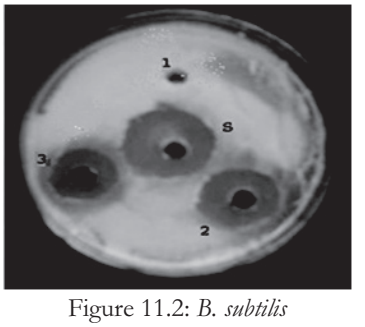

Figure 11.2: *B. subtilis*

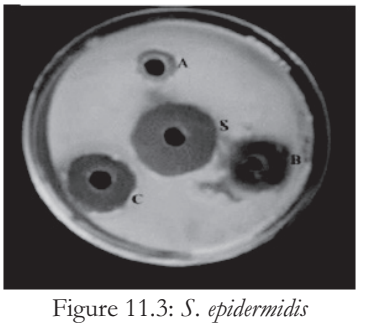

Figure 11.3: *S. epidermidis*

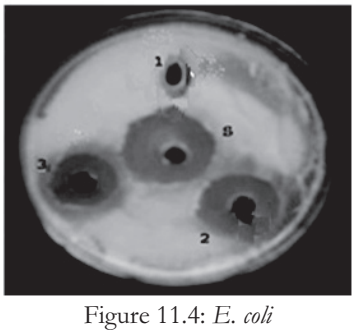

Figure 11.4: *E. coli*

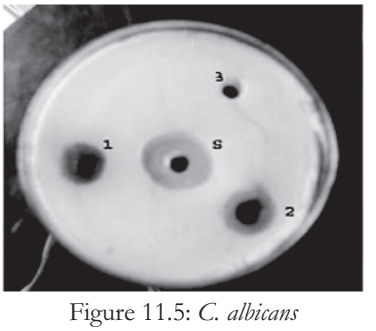

Figure 11.5: *C. albicans*

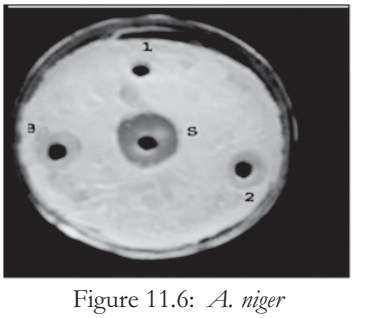

Figure 11.6: *A. niger*

Figure 1. Zone of inhibition of EEA against micro-organisms where **1**: 0.5 mg/ml **2**: 1 mg/ml **3**: 1.5 mg/ml.

antibacterial activity, while the extraction with the petroleum ether gave zone of inhibition equal to 6–15 mm, MIC value was found to be 600 μg/ml and MBC value was found to be 900 μg/ml, respectively. The antifungal activity of the fluconazole and petroleum ether extract was found to be nearly similar since fluconazole is the drug of choice for *Candida albicans*.

The triterpenes while steroids in petroium ether extracts found by Salkowaski test and Libermans test. MIC value for the extract of *Echinacea angustifolia* was 1 mg/ml, 1.5 mg/ml for various viz. *S. aureus, E. coli*, *Staphylococcus epidermidis* and *Candida albicans* activity is due to cichoric acid, echinacoside steroids, terpenoids, flavonoids and Alkaloids, isotussilagine, tussilagine, Alkylamides (alkamides), echinacein was reported.[14] The current work has shown that *Echinacea angustifolia* is a potential source of antimicrobial agents and its activity against various bacteria and fungus may be sufficient to perform further studies for isolation and identification for active principles.

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