Chemical Examination and Biological Studies on the Bark of *Crataeva nurvala* Buch.-Ham.

G. Venkateswara Rao*, T. Annamalai, T. Mukhopadhyay

M/s. Cavinkare Research Centre, 12, Poonamalle Road, Ekkattuthangal, Chennai 600 032, India

ABSTRACT

In continuation of our investigation on bioactive molecules, the melanin promotion activity-guided fractionation of the methanolic extract of the bark of *Crataeva nurvala*, yielded two known triterpenoid derivatives, lupeol (**1**) and lupenone (**2**). The structure of the compounds were established by physical and spectral data (UV, IR, ^{1}H, ^{13}C NMR and Mass). The compounds were shown potent melanin promotion activity when compared with standard control compound, 3-isobutyl-1-methylxanthine (IBMX).

Key words: *Crataeva nurvala*, triterpenoids, melanin promotion activity.

INTRODUCTION

Crataeva nurvala Buch.-Ham., belongs to the family Capparaceae. It is a medium sized, ornamental, deciduous tree, 6-10 m tall, found either wild or cultivated throughout India. The stem bark is bitter astringent and acrid. In Indian medicine, the stem bark is being widely used as single drug or in compound formulations for the treatment of urinary disorders including urolithiasis, prostatic hypertrophy, blood purifier, skin infections, neurogenic bladder and chronic urinary infections. The stem-bark is also considered anti-inflammatory and also reported to stimulate appetite, bile secretion and bowel movement.[1] It is externally applied to abcess, boils and lymphadenopathy. The leaves are also bitter, acrid, stomachic, depurative, anti-inflmmatory, tonic, antiperiodic and expectorant.[2] The decoction of leaves and stem is used as a remedy for desentery and diarrhoeal diseases by the natives of Taiwan. It is also used as an anticonvulsant in the folklore medicine of the Philippines.[1] Several reports of *C. nurvala* have demonstrated the presence of steroids, terpenoids, fatty alcohols and acids, flavonoids.[3-7] In continuation of our interest on the isolation of bioactive compounds from medicinal plants for cosmetic applications,[8-13] we have undertaken chemical examination of the bark of *C. nurvala*. The present study describes the isolation of two triterpenoids: lupeol (**1**) and lupenone (**2**) from the bark of *C. nurvala*. The crude extract, its fractions and isolated compounds were analysed for melanin promotion activity in B16F10 cells and found that the isolated compounds were shown potent melanin promotion activity.

Address for correspondence:
E-mail: rao.gv@cavinkare.com

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MATERIALS AND METHODS

General

Melting points reported are uncorrected. The 400 MHz NMR spectra were recorded on a Bruker AMX 400 in $CDCl_3$ or CD_3OD with TMS an internal standard. The ^{13}C NMR spectra were recorded at 100 MHz in $CDCl_3$ and CD_3OD. IR spectra were recorded on a Shimadzu IR prestige 21; UV spectra were recorded on Shimadzu UV spectrophotometer; GC-MS were on a Jeol SX 102/DA 6000 mass spectrometer. TLC was performed on pre-coated silica gel 60 F_{254} plates (Merck) and the spots were visualized by exposure to iodine vapour or spraying with 5% sulphuric acid in methanol followed by heating the plate at 110ºC for 5 min. The IBMX was purchased from the M/s. Sigma-Aldrich, USA

Plant material

The bark of *C. nurvala* was obtained from bazaar and was authenticated by Dr. P. Santhan, botanist, M/s. Durva Herbal Centre, Chennai. A voucher specimen was deposited in M/s. CavinKare Research Centre, Chennai, India.

Extraction and Isolation

Air-dried bark of *C. nurvala* (1.8 kg) was crushed and coarsely powdered, subjected to an extraction with methanol (3 L)

by using a soxhlet apparatus. After evaporation of the solvent under reduced pressure, 46g of crude methanolic extract was obtained. The crude methanolic extract (45.5g) was suspended in methanol : water (2:8) and partitioned with ethyl acetate and saturated n-butanol to get corresponding fractions 16.1 g and 4.2 g respectively. The EA fraction showed good melanin promotion activity, where as n-butanol fraction showed less activity.

After TLC analysis, the dark brown residue from ethyl acetate fraction (16.0 g) was subjected to silica gel column chromatography, eluted with hexane: ethyl acetate (1:1, 3:1) and ethyl acetate. The homogeneous fractions were combined based on TLC and divided into three major fractions A (5.6 g), B (6.3 g) and C (1.5 g). Fraction A was further purified by silica gel column using a solvent mixture hexane: ethyl acetate (9:1, 7:3) yielded one compound which was re-crystallized with hexane: ethyl acetate to get colorless crystals **2** (230 mg). Fraction B came as brown color solid, which was directly re-crystallized with ethyl acetate and methanol to get colorless amorphous compound **1** (4.65 g).

Compound **1:** Colorless amorphous powder, mp: 201-202°C; UV ($CHCl_3$, γ_{max} in nm): 215; IR (KBr, ν_{max} in cm^{-1}): 3340 (hydroxyl), 2850, 1620, 1400, 980, ^1H NMR (CD_3OD): δ 0.75 (3H, s, 24-H), 0.82 (3H, s, 28-H), 0.86 (3H, s, 25-H), 0.94 (3H, s, 27-H), 0.95 (3H, s, 23-H), 1.04 (3H, s, 26-H), 1.69 (3H, s, 30-H), 2.40 (1H, ddd, *J* = 5.6, 110.11.0, 19-H), 3.12 (1H, dd, *J* = 5.1, 11.5 Hz), 4.55 & 4.67 (each 1H, d, *J* = 1.3Hz, 29-H), GC-MS: M/z 426 (36%), 411 (16%), 311 (15%), 257 (11%), 218 (53%), 189 (74%), 135 (79%), 121 (77%), 109 (91%), 95 (100%).

Acetylation of **1**: To a solution of **1** (50 mg) in pyridine (2 ml), Ac_2O (2 ml) was added and the mixture kept for 24 hr at room temperature. After usual work-up yielded mono acetate derivative, **1a** (45 mg).

Compound **1a:** Colorless powder, mp: 164-66°C. ^1H NMR ($CDCl_3$): δ 0.78 (3H, s), 0.83 (3H, s), 0.84 (3H, s), 0.85 (3H, s), 0.94 (3H, s), 1.02 (3H, s,), 1.68 (3H, s), 2.04 (3H, s), 4.46 (1H, dd, *J* = 6.1, 10.1 Hz), 4.57 (1H, s), 4.68 (1H, s). GC- MS: M/z 468 (26%), 453 (12%), 408 (8%), 311 (15%), 218 (42%), 204 (46%), 189 (100%), 121 (80%), 109 (84%), 95 (92%), 81 (71%), 69 (59%).

Compound **2**: Colorless crystals, mp: 164-66°C; UV ($CHCl_3$, γ_{max} in nm): 214; IR (KBr, ν_{max} in cm^{-1}): 2937, 1705 (carbonyl), 1448, 1382, 1016, 869; ^1H NMR ($CDCl_3$): δ 0.80 (3H, s, 28-H), 0.93 (3H, s, 25-H), 0.96 (3H, s, 27-H), 1.03 (3H, s, 23-H), 1.07 (6H, s, 24 & 26-H), 1.68 (3H, s, 30-H), 4.58 (1H, m, 29-H), 4. 70 (1H, d, *J* = 2.2 Hz, 29-H).

Melanin promotion assay

The melanin promotion activity[18] of crude methanolic extract, its fractions, isolated compounds and IBMX (control) were studied in cell lines (B16F10 melanoma cells). The assay method is most precise and reliable. The compound lupeol (**1**) showed potent activity by producing more melanin in the cells than its oxidized compound, lupeonone (**2**). Similarly, another derivative of lupeol, lupeol acetate showed less activity than its parent compound (Table 2)

RESULTS AND DISCUSSION

Repetitive chromatographic steps of the ethyl acetate fraction of the dried bark of *C. nurvala* yielded two known triterpenes [Figure 1].

Compound **1** was obtained as colorless amorphous powder, mp: 201-202°C. It was readily recognized as triterpene derivative from its preliminary NMR spectral data. Its molecular formula was established as $C_{30}H_{50}O$ by GC-MS, M^+ 426. Its IR spectrum showed the presence of hydroxyl group (3340 cm^{-1}) and its UV spectrum showed no characteristic absorption. The ^1H-NMR spectrum clearly showed triterpenoid pattern with six methyl singlets in the up-field at δ 0.75 (Me-24), 0.82 (Me-28), 0.86 (Me-25), 0.94 (Me-27), 0.95 (Me-23), 1.04 (Me-26) and one more methyl appeared as a sharp singlet at δ1.69 (Me-30). The down field shift for Me-30 indicated the presence of double bond between C-20 and C-29. Further, the proton spectrum showed two peaks at δ 4.55 & 4.67 (each 1H, d, *J* = 1.3 Hz, H-29) suggesting the presence of exomethylene protons. Additionally, the spectrum showed signal at 3.12 (1H, dd, *J* = 11.3, 5.1Hz, H-3) which suggested the presence of secondary hydroxyl group.

The ^{13}C NMR spectrum (100 MHz, CD_3OD) [Table1] showed 30 carbon signals. Out of which two double bond carbon signals at δ 150.4, 108.6 and methyl signal at δ19.3 indicates the presence of isopropenyl group in the molecule. The spectrum also clearly showed only one oxygenated carbon at δ 78.1. Further the spectrum showed six methyl, ten methylene, five methine and five quaternary carbon signals. Based on the above spectral data and in comparison with literature data, the structure of the compound **1** has been established as lupeol.[7, 14-16]

Compound **2** was isolated as colorless crystals from hexane: ethyl acetate, mp:168-70°C. It was readily recognized as triterpene derivative from its preliminary NMR spectral data. Its molecular formula was established as $C_{30}H_{48}O$ by GC-MS, M^+ 424. Its IR spectrum showed the presence of carbonyl group (1705cm^{-1}) and its UV spectrum showed

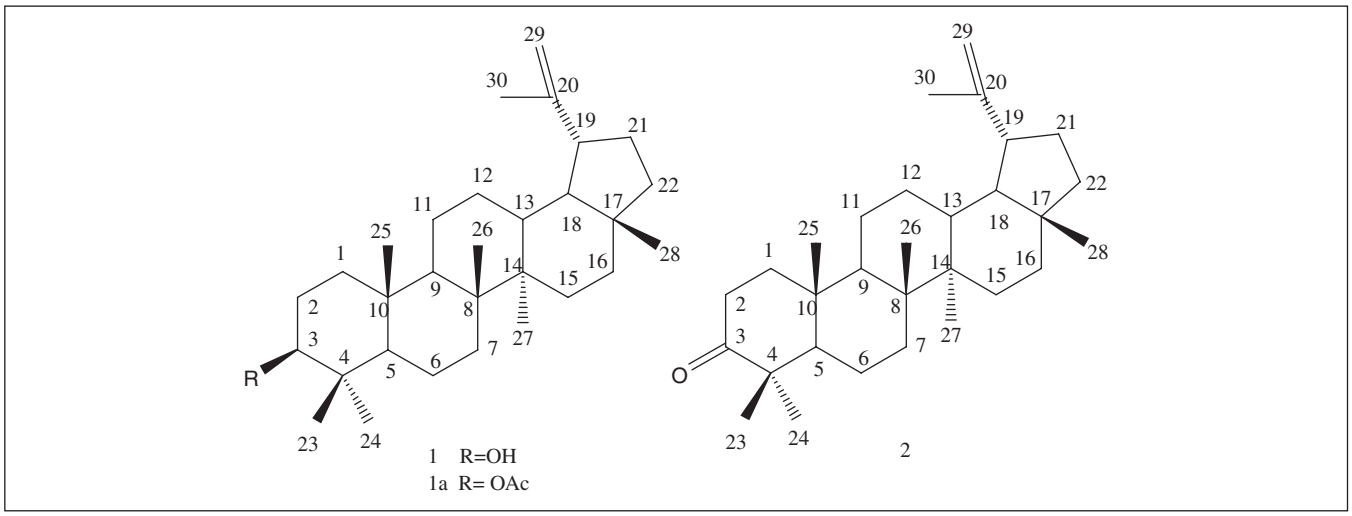

Figure 1: Compounds from *C. nurvala*

Table 1: ^{13}C NMR Spectroscopic data for compound 1 in CD_3OD and compound 2 in $CDCl_3$ (100 MHz)

Position	1	2
C-1	38.4	34.1
C-2	27.1	39.9
C-3	78.1	218.1
C-4	38.6	47.2
C-5	55.3	54.8
C-6	17.9	19.6
C-7	34.0	33.5
C-8	40.5	40.7
C-9	50.4	49.9
C-10	36.8	36.8
C-11	20.5	21.4
C-12	25.0	25.1
C-13	38.0	38.1
C-14	42.4	42.9
C-15	27.1	27.3
C-16	35.2	35.4
C-17	42.5	42.8
C-18	48.3	48.1
C-19	47.9	47.2
C-20	150.4	150.8
C-21	29.4	29.7
C-22	39.5	39.6
C-23	27.9	26.6
C-24	15.1	21.2
C-25	16.0	15.9
C-26	15.9	15.8
C-27	14.5	14.4
C-28	17.9	18.0
C-29	108.6	109.3
C-30	19.3	19.2

Table 2: *In-vitro* Melanin promotion activty

Compound/Fraction	Concentration (μg/ml)	%Melanin promotion
Methanolic extract	75	97.5
Ethyl acetate fraction	25	108
n-Butanol fraction	35	20
IBMX (Control)	15	70
Lupeol (**1**)	15	401
Lupenone (**2**)	15	150
Lupeol acetate (**1a**)	15	245

no characteristic absorption. The ^1H-NMR spectrum clearly showed triterpenoid pattern with six methyl singlets at δ 0.80 (Me-28), 0.93 (Me-25), 0.96 (Me-27), 1.03 (Me-23), 1.07 (Me-24), 1.07 (Me-26), a vinyl methyl signal at δ 1.68 (Me-30) and an exomethylene group signals at δ 4.58 (1H, m, H-29), 4. 70 (1H, d, *J* =2.2 Hz, H-29).

The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) [Table1] showed clearly 30 carbon signals. Out of which one carbonyl signal at δ 218.7 and two double bond signals at δ 150.8 and 109.3. No oxygenated carbon signal was observed in the spectrum. By comparing the compound **1** and **2** spectral data, the former contains hydroxyl group and later contains keto group in the molecule, remaining all other signals are more or less same. The compound **2** might be an oxidized version of compound **1**. The structure of compound **2** was deduced from the comparison of its spectral with those of literature and identified as lupenone.[17]

CONCLUSION

The present investigation has helped to identify the major source for lupeol and also potent melanin promoter. Lupeol fatty acid esters have been reported for melanogenesis

promotion activity by Japanese Scientists but not lupeol and lupenone. The study of melanin promotion activity is first time for this plant.

ACKNOWLEDGEMENT

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Pharmacognostic, Physicochemical and Phytochemical Studies on *Carica papaya* Linn. Leaves

Zunjar V[1], Mammen D[2], Trivedi BM[3]*, Daniel M[4]

[1, 2, 3]*Department of Chemistry, M.S. University of Baroda, Vadodara-390 002,*
[4]*Department of Botany, M.S. University of Baroda, Vadodara-390 002.*

ABSTRACT

Papaya (*Carica papaya* Linn.) is commonly known for its food and nutritional values throughout the world. The medicinal properties of papaya fruit and other parts of the plant are also well known in traditional system of medicine. During the last few decades considerable progress has been achieved regarding the biological activity and medicinal application of papaya and now it is considered as valuable nutraceutical fruit plant. The present study deals with the microscopic evaluation of leaves of *Carica papaya* Linn., to establish the salient diagnostic features for the leaf. The leaf shows abundant sphaeraphides and rhomboidal calcium oxalate crystals. The leaf shows no trichomes. Micromorphological studies conducted on the leaf gave value of stomatal index to be 31.56 ± 3.41, vein termination number 3-4, and palisade ratio to be 12.65 ± 1.57. The leaf shows a continuous network of veins. Histochemical tests performed indicate the presence of alkaloids and starch. Powder study shows the presence of sphaeraphides, starch grains and rhomboidal calcium oxalate crystals, apart from regular characters such as stomata and spiral xylem. Physicochemical parameters such as extractive values, ash values and moisture content have also been studied for the leaf. The results of this study could be useful in setting some diagnostic indices for identification, authentication and preparation of the monograph of the plant.

Key words: *Carica papaya*, anatomy, micromorphology, sphaeraphides

INTRODUCTION

Carica papaya Linn. (Caricaceae) is a fast-growing, semi-woody tropical herb reaching 3-10 m in height. The fleshy stem is single, straight and hollow and contains prominent leaf scars. Papaya exhibits strong apical dominance rarely branching unless the apical meristem is removed, or damaged. *Carica papaya* contains many biologically active compounds. Two important compounds are chymopapain and papain, which are supposed to aid in digestion. Papain also is used to treat arthritis. The level of the compounds varies in the fruit, latex, leaves, and roots. Since, each part of papaya tree possesses economic value; it is grown on commercial scale.[1]

The present investigation of *Carica papaya* Linn leaves was taken up to establish pharmacognostic profile which will help in crude drug identification as well as standardization of the quality and purity of the drug in crude form.The present study comprises the macroscopical, microscopical and phytochemical studies of the leaves of *Carica papaya* Linn., since no proper report is available on the pharmacognosy and anatomy of the leaf of the plant.

The ash content of a crude drug is the inorganic residue remaining after incineration. It includes not only the inorganic salts, e.g. Calcium oxalate, occurring naturally in the drug; but also inorganic matter from external sources.[2] Four types of ash values used in routine pharmaceutical analysis are Total ash content, Acid insoluble ash, Water soluble ash and Sulphated ash. **Total ash content** - A figure for total ash content is useful when the contamination with calcium oxalate is very little. If more quantity of calcium oxalate is present, then the value for the acid insoluble ash is a better criterion of purity. **Acid insoluble ash** - Crude drugs containing larger quantity of calcium oxalate, can give variable results depending upon the conditions of ignition. Treatment of the ash with Hydrochloric acid leaves virtually only silica. Hence acid insoluble ash forms a better test to detect and limit excess of soil present as an impurity

Address for correspondence:
E-mail: trivedibhavna@yahoo.com

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in the drug, than does the total ash. **Water soluble ash** - It is a measure of detection of water soluble impurities in drug or raw material. **Sulphated ash** - The determination of sulphated ash is widely used to control the extent of contamination by non-volatile inorganic impurities in organic substances. For sulphated ash, the substance is ignited using small quantities of sulphuric acid, which decomposes and oxidises the organic matter, leaving a residue of inorganic sulphates. Reproducible results are readily obtained in this determination than in total ash determination, due to the higher stability of metal sulphates.

Plants and their parts such as roots, stems, barks, leaves, flowers, fruits, seeds and exudates constitute major portion of drugs used in traditional herbal systems of medicine. The therapeutic efficiency of the drugs used in these systems greatly depends on the use of proper and genuine raw materials. Due to this reason, the assurance of safety, quality and subsequent efficacy of the medicinal plants and herbal products has now become a major and key issue. Both the general public as well as health care professionals require updated authoritative information on the safety and efficacy of medicinal plants.[3] Pharmacognosy includes mainly micromorphological, anatomical and powder studies, though TLC fingerprinting also is an essential feature.

Ayurvedic Pharmacopoeia Committee constituted by the Department of AYUSH recently has introduced certain quality parameters for the plants used in Indian System of Medicines. It now is important for the manufacturers of these drugs, to confirm that their raw materials are identified to be genuine. It is a mistaken notion that the classical procedures are obsolete and only chemo-taxonomical profile can provide a true identity of the drug and thus distinguish them from substitutes and adulterants. It is observed that the classical procedure of powder microscopy is still a useful tool for species identification and can play a pivotal role in the authentication of the drug material.[4]

MATERIALS AND METHODS

Collection and authentication of plant material

Carica papaya Linn. Collected in August 2010 from Vadodara, Gujarat, India, was identified and authenticated at The Department of Botany, M. S. University of Baroda, Gujarat, India. The voucher specimen of this plant (No. BARO/2010/51) was deposited at the Herbarium, BARO, Department of Botany, M. S. University of Baroda. The plant material was washed, shade dried for a day and then dried completely in an oven at 38^0C. The plants were coarsely powdered using a rotary grinder and stored in airtight plastic containers, and then used for phytochemical analysis. Fresh leaves were used for micromorphological and anatomical studies.

Physicochemical parameters

Physicochemical parameters such as extractive values, ash values and moisture content, were performed as per the official standard procedures.[5,6]

Anatomy

Sections of fresh leaf were subjected to staining using Safranine (1% in water). The slides were then mounted and sealed using DPX. The slides were then observed under the microscope and the sizes of various cells observed in the tissues were measured using an ocular micrometer. The least count of the micrometer was calculated for this purpose. The sections were photographed under a Leica DM 2000 microscope connected to a digital Canon camera.

Micromorphology

Fresh leaves were washed and small fragments of leaves were taken from the middle region of the lamina of mature leaves. For anatomical studies, sections of 10-12 µm thick were prepared and stained with Safranine (0.5%) in water and then mounted in 50% glycerine. Clearing of leaf was done to study the venation pattern. Washed leaf fragments were first boiled in 90% alcohol for about 3-5 minutes to remove chlorophyll, then washed 2-3 times with water, then boiled again with 10% KOH solution for 2-3 minutes and washed 4-5 times with water. The epidermal layer was peeled off using the help of pointed needle and forceps and was washed in water, stained with Safranine (0.5%). The margins of the cover slips were sealed with DPX, and the slides were observed under the microscope. Stomatal index, palisade ratio, vein termination number and vein islet number were then calculated using standard procedures.[7,8]

Powder studies

Completely dried plant material was finely powdered and sieved through BSS mesh No. 85. The fine powder obtained was stained using Safranine in water. The stained powder was mounted on a slide and observed under a microscope to locate and identify the characters present. The characters observed were photographed under a Leica DM 2000 microscope connected to a digital Canon camera.

Histochemical tests

Specific reagents for identification of important classes of compounds were prepared according to procedures prescribed in the WHO guidelines. Sections of midrib as well as cleared sections of the lamina were treated with these reagents and mounted on slides for observation under a microscope.[6]

Preliminary phytochemical screening

The shade dried and coarsely powdered leaves were extracted successively with different solvents by using soxhlet

apparatus and analysed using simple chemical tests for preliminary screening of various groups of phytoconstituents such as alkaloids, flavonoids, phenolic acids, sterols, cardiac glycosides, tannins, and so on, as per WHO guidelines.[6,9]

RESULTS AND DISCUSSION

Physicochemical parameters

Physicochemical parameters like percentage of moisture content, total ash, acid insoluble ash, water soluble ash, ethanol soluble extractive and water soluble extractive were determined and depicted in **Table 1**.

Anatomy

The transverse section of *Carica papaya* Linn. leaf (**Plate 1**) shows that the leaf was dorsiventral. The mid rib portion was almost spherical with a large portion on the lower side. The leaf was glabrous without any hairs on either side. The lamina segregated into upper epidermis, mesophyll and lower epidermis. Epidermis consist of barrel shaped cells (8 μm × 4 μm) below which there were layer of collenchyma. Vascular bundles was of collateral closed arrangement. Vascular bundles were arranged in a ring with two vascular bundles positioned one on the upper side and other on the lower side. The other bundles were small and represented by 3-4 tracheids. Xylem consist of tracheids (3.96 μm - 10.56 μm) only and vessels were absent. The phloem was represented by radially elongated patches of cells (1.98 μm - 3.3 μm) separated from one another by parenchyma cells, some of which contain chlorophyll. Between the bundles and epidermis was a broad band of collenchyma. The central portion was represented by a hollow region. Articulated laticiferous canals accompany the vascular bundles of the veins and extend into the surrounding mesophyll. A large number of sphaeraphides (2.64 μm - 6.6 μm) were seen throughout the collenchymatous cortex and parenchymatous ground tissue. In the laminal region mesophyll was differentiated into upper palisade and lower spongy. Palisade was two layered, each cell having dimensions (10 μm × 2 μm). The spongy tissue consisted of 5-6 layers of closely packed mesophyll cells (6 μm × 4 μm). The section also showed the presence of prismatic calcium oxalate crystal.

Table 1: Values obtained for the proximate analysis of *Carica papaya*

Parameter	%w/w
Total ash	17.4
Acid insoluble ash	13.3
Water soluble ash	3.25
Sulphated ash	27.05
Ethanol soluble extractive	8.4
Water soluble extractive	17.7
Moisture content	5.70

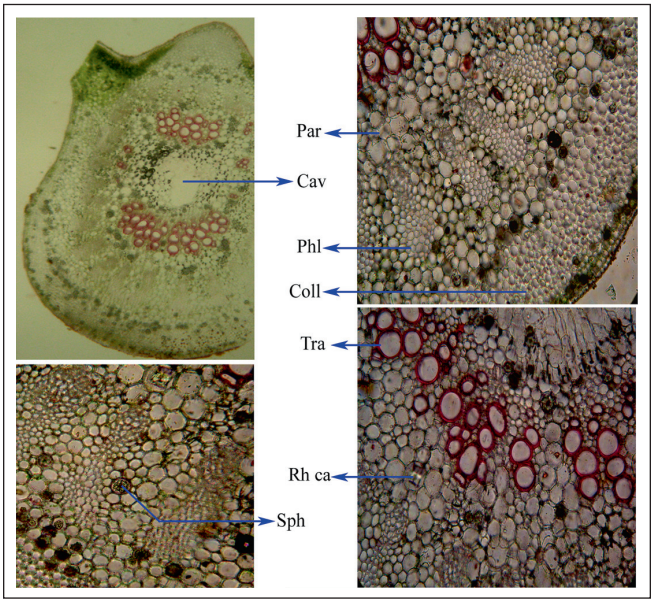

Plate 1: Transverse section of leaf of *Carica papaya*, showing the presence of **Par**- parenchyma, **Cav**- Hollow cavity, **Phl**- phloem, **Coll**- collenchyma, **Tra**- tracheids, **Rh ca**- Rhomboidal calcium oxalate crystals, and **Sph**- sphaeraphides.

Micromorphology

Vein termination number in *Carica papaya* leaf showed values close to 3-4, stomatal index was calculated to be 31.56 ± 3.41, while the palisade ratio was calculated as 12.65 ± 1.57. The leaf showed a continuous network of veins hence vein islet number was found to be zero. Anomocytic stomata were found restricted only to the lower epidermis (**Plate 2**).

Powder study

Sphaeraphides, starch grains and rhomboidal calcium oxalate crystals form diagnostic characters in the leaf powder of *Carica papaya*. Other regular characters like stomata and spiral xylem were also found in the powder (**Plate 3**).

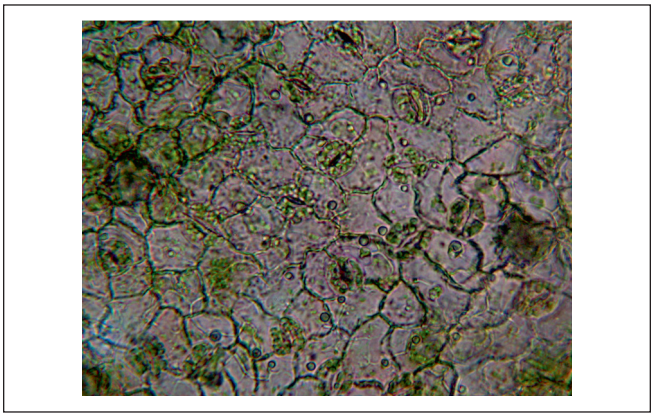

Plate 2: Lower epidermis of leaf of *Carica papaya* showing the presence of anomocytic stomata

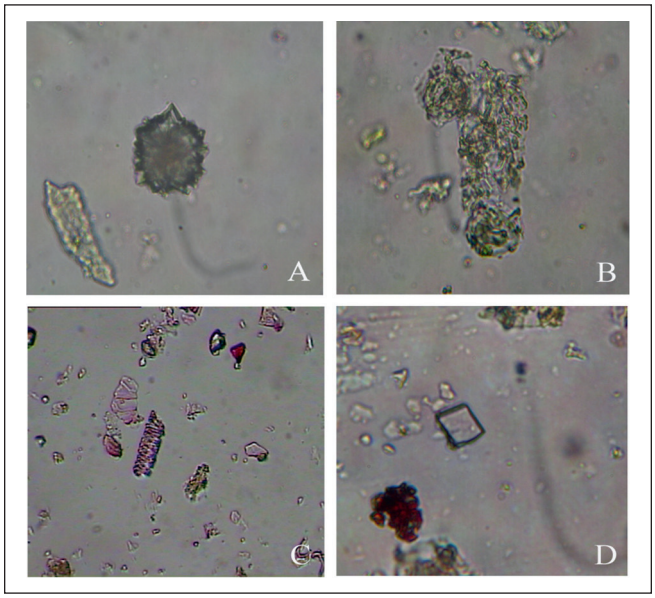

Plate 3: Powder of *Carica papaya* showing **A**- Sphaeraphide, **B**- Stomata, **C**- Spiral xylem, starch grains, **D**- Rhomboidal calcium oxalate crystal.

Table 2: Results of histochemical tests performed of sections of *Carica papaya*

Cells contents	Reagent used	Observations
Lignified cell walls	Phloroglucinol + HCl	+
Calcium oxalate	HCl	+
Starch	Iodine	+
Tannins	$FeCl_3$	–
Cellulose	Iodine + H_2SO_4	+
Alkaloids	Dragendorff	+
Aleurone grains	Iodine	–

Histochemical tests

The results of histochemical detection are furnished in **Table 2**.

Preliminary phytochemical screening

Preliminary phytochemical screening revealed the presence of carbohydrates, amino acids, saponin glycosides,iridoids, flavonoids, phenolics, and alkaloids (**Table 3**).

Table 3: Results of phytochemical tests performed on powder of *Carica papaya*

Group of phytoconstituents	Observations
Carbohydrates	+
Gums and mucilages	–
Proteins	+
Amino acids	+
Steroids	–
Cardiac glycosides	+
Anthraquinone glycosides	–
Saponin glycosides	+
Flavonoids	+
Alkaloids	+
Tannins	+
Phenolics	+
Iridoids	+
Anthocyanins	–

CONCLUSIONS

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 pharmacopeia, 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 and physicochemical parameters. The present work is undertaken to produce some pharmacognostical standards for *Carica papaya*. The above studies provide information in respect of their identification, chemical constituents and physicochemical characters which may be useful for pharmacognostical study and standardization for the plant.

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Extraction and Characterization of Tamarind Seed Polysaccharide as a Pharmaceutical Excipient

Rupali Singh*, Rishabha Malviya, Pramod Kumar Sharma

Department of Pharmaceutical Technology, Meerut Institute of Engineering and Technology Baghpat Crossing, Delhi Roorkee Highway, NH-58, Meerut-250005, U.P. India

ABSTRACT

Objective: The objective of the present work was extraction of polysaccharide from tamarind seed and further characterization as pharmaceutical excipient. Study includes phytochemical screening, micromeritic properties. Work also emphasize to study gelling properties of extracted polysaccharide. **Methods:** Water based extraction procedure was used to extract polysaccharide from tamarind seed. Pharmacopoeial procedures were used to study the micromeritic properties, solubility, organoleptic properties and pH. Different concentration based solution were prepared to evaluate gelling properties of seed polysaccharide. **Key findings:** Results obtained from the study showed that used procedure was efficient to extract gum from tamarind seed. Obtained results easily predict the fact that extracted polymer can be used as pharmaceutical excipient in terms of micromeritic properties and flow behavior. It was also found that obtained gum showed gelling behavior at 8% w/v solution of water. **Conclusions:** It can be concluded from whole study that tamarind seed polysaccharide can be an important pharmaceutical excipient for solid. Obtained results also showed that extracted seed polysaccharide may be used as natural gelling agents in different pharmaceutical formulations.

Key words: tamarind gum, rheological studies, natural polymers, polysaccharides, excipient.

INTRODUCTION

Natural polymers are obtained in the form of macromolecules. The natural polymers demands in the households, agriculture, food industries and in packaging and it also help in reducing the environmental pollution and resulting in disposal in landfills. Natural polymers are act as an environment cleaner, renewable and help in recycling of global carbon. The natural gums are biodegradable, nontoxic and biocompatible in nature and swells when comes in contact with the aqueous media so it has been used in the preparation of sustained release or controlled release types of dosage form.[1, 2] In the present investigation it shows that plant polysaccharide have been useful for the construction of specific drug delivery systems.[2] The polysaccharide that is present in tamarind gum is known as tamarind seed polysaccharide (TSP). Gum is present in the tamarind seed and it is a hydrophilic polymer and had been used as gelling, thickening, suspending and emulsifying agents.[3, 4, 5, 6] Gum consisted 65% of the seed components.[7, 8, 9] It is used as a thickening, stabilizing and gelling agents in food.[10] The gum can also be act as a binder in pharmaceutical tablets, as a humectants and emulsifier in the different types of formulations.[11] This shows that the Tamarind gum is highly viscous, mucoadhesive and biocompatible in nature. Now the regular research is going on in field of natural occurring biocompatible polymeric material in designing of dosage form for oral controlled release administration. The Tamarind gum has a widest scope in the pharmaceutical industries and it is act as a binder in tablet dosage form, ocular drug delivery system and in sustained release drug delivery systems. It is a novel mucoadhesive polymer, can be used in the delivery system for the ocular administration of hydrophilic and hydrophobic antibiotics.[12]

Address for correspondence:
E-mail: rupalisingh04py43@gmail.com
Ph.: +919758395798

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METHOD

Isolation of gum from tamarind seed

Take the tamarind seeds and peel out the outer cover and obtaining the white part of seeds and crush them. The crushed seeds of *Tamarindus indica* were soaked in water for 24 h and then take the muslin cloth and the soaked seeds were put into it for the release of gum from it. The marc was removed from the gum and

equal quantity of absolute ethyl alcohol was added to precipitate the gum and the gum was separated by filtration. The marc was not discarded because it was sent for multiple extractions with decreasing quantity of extracting solvent i.e., water with the increase of number of extractions. The isolation was continued until the material was free of gum. The separated gum was dried in hot air oven at temperature 40ºC. Then the dried gum was powdered and stored in airtight containers at room temperature.[11]

Physicochemical characterization of gum

Identification tests for carbohydrates, proteins, mucilage and gums:

Aqueous solution of extracted gum was used for chemical characterization. Test for carbohydrates, proteins, mucilage, alkaloids, fats, tannins, amino acids and gums were perform according to standard procedure.[13,14]

Organoleptic evaluation of isolated gum:

The isolated gum was characterized for organoleptic properties such as color, odor, taste, fracture and texture.

Solubility behavior gum:

One part of dry gum powder was shaken with different solvents and the solubility was determined.

pH of gum:

The gum was weighed and dissolved in water separately to get a 1% w/v solution. The pH of solution was determined using digital pH meter.[14]

Swelling index:

The swelling index is the volume (in ml) taken up by the swelling of 1 g of test material under specified conditions. The swelling index of the pectin was determined by accurately weighing 1 g of pectin, which was further introduced into a 25 ml glass-Stoppard measuring cylinder. 25 ml of water was added and mixture was shaken thoroughly every 10 min for 1 h. It was then allowed to stand for 3h at room temperature. Then the volume occupied by gum, was measured. The same procedure was repeated thrice and the mean value was calculated.[14]

Bulk density and bulkiness:

The inverse of bulk density is called as bulkiness. Accurately weighed quantity of (50 g) was introduced into a graduated measuring cylinder. The cylinder was fixed on the bulk density apparatus and the volume occupied by the powder was noted. Then, the powder was subjected to tapping in a bulk density apparatus until constant volume was obtained. The final volume (bulk volume) was noted.[15, 16]

Powder flow property:

The flow characteristics were measured by angle of repose. The experiment was repeated thrice. Using the readings and the formula, the angle of repose was calculated.[15,16]

Powder compressibility:

This property is also known as compressibility. The finely powdered gum (5 g) was transferred into a measuring cylinder and calculations were done using bulk density apparatus.[15,16]

Evaluation of gelling properties:

The gelling property of the tamarind gum was evaluated with preparing different concentration based gels of polymer. In this study initially 3%, 4% and 5% of solutions of the gum were prepared but the gel formation was not appeared. Further 6%, 8%, 10% and 12% solution were prepared and it was found that gelling property of polymer was initiated with 8% solution at 25ºC. The viscosity of the prepared gels was estimated using Brookfield Rheometer (Model No. R/S- PI).

Inter day measurementof viscosity:

Interday measurement of viscosity was carried out with an aim to evaluate effect of relatively long time on the viscosity of prepared gel. All the measurements were carried out at 25ºC and 100 shear rate.

RESULTS AND DISCUSSION

After extraction of the gum and then further precipitation by the ethyl alcohol the yield of tamarind gum was obtained 18.39% w/w. isolated gum was then used for identification test. This showed the presence of carbohydrates in the sample powder of the gum. The confirmatory test of gum was done when it gave negative test for mucilages, tannins, alkaloids and proteins. This can be considered as a proof for purity of the isolated gum as depicted in Table 1.

Table1: Chemical characterization of isolated gum

Tests	Present/Absent
Carbohydrates	+
Hexose Sugar	+
Monosaccharide	–
Proteins	–
Fats and oils	–
Tannins	–
Alkaloids	–
Amino acids	–
Mucilage	–
Gums	–

+Present; –Absent.

Organoleptic properties of gum were found to be accepted. Gum was odorless having the characteristics taste. Gum was light brownish in color with rough texture and rough fracture. Ash values are an important parameter to characterize the natural excipients in the gum. The solubility behavior of any excipient is an important parameter to determine its suitability in different formulations. Isolated gum was also used to study the solubility behavior. It was found that gum swells to form gel in cold water while completely insoluble in organic solvent such as benzene, ether, chloroform, n–butanol, methanol and ethanol. The pH value of 1% solution of the gum was found to be slightly acidic (6.7). So it should not show the mucosal irritation when used in the formulations for the oral preparations. Physical characterization of gum was carried out for the bulk density, bulkiness, true density total porosity, powder flow behavior and surface characteristics of the powder using SEM. The bulkiness value indicated that powder is 'light' in nature. The total porosity has been correlated with dissolution rate. It has been found that higher the porosity the faster the dissolution rate. The gum has exhibited the good flow characteristics. Values of micromeritic properties and flow behavior were showed in Table 2.

Interday measurement of viscosity

Values obtained from the viscosity measurement of prepared gel was shown in Table 3. It was found that viscosity of different gels was not change significantly from one day to another.

Table 2: Micromeritic study and flow properties of gum

Parameters	Values
Angle of repose	16.35
Carr's index	4.32%
True density (gm/ml)	0.992
Bulk density (gm/ml)	0.943
Bulkiness	1.060

Value with "±" showed standard deviation of triplicate readings

Table 3: Viscosity measurement of prepared gel in different days

Days	Viscosity (Pa)			
	6%	8%	10%	12%
1	6.79	11.9	8.21	9.41
2	7.11	11.2	8.62	9.83
3	7.98	11.4	8.44	9.54

CONCLUSIONS

It was found that polysaccharide can be successfully extracted from tamarind seed using water based extraction procedure. Extracted polysaccharide has properties to be used as pharmaceutical excipient. It was also predicted from the study that extracted polysaccharide can be used as a gelling agent in different pharmaceutical preparations.

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Pharmacognostic evaluation of the rhizomes of *Curcuma zedoaria* Rosc.

Sharad Srivastava, Shanta Mehrotra and AKS Rawat*

Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute (CSIR), Lucknow-226001, India

ABSTRACT

Curcuma zedoaria Rosc. (Family-Zingeberaceae), commonly known as '*yellow zedoary*' is used in India system of medicine since time immemorial. The plant is found well in eastern Himalayas, Chittangang, Bengal, Kerala, Konkan and often cultivated throughout India. It is aromatic, pungent, bitter and useful in flatulent colic and debility of the digestive organs and also used as an ingredient in bitter tincture of zedoary and anti-periodic pills. A paste of rhizome is useful externally for cuts, wounds, itching and in sprains. A detailed phytochemical evaluation of its rhizome showed moisture content 83.22%, total ash 6.64%, acid insoluble ash 0.64%, alcohol soluble extractives 15.53%, water soluble extractives 18.96%, sugar 12.51% and starch 15.70%. A study of its volatile content also has been done indicating 2.8% of total volatile oil. These findings will be very useful for the identification of the species which may be useful to pharmaceutical industries for the quality control of the commercial samples.

Key words: *Curcuma zedoaria*, HPTLC, Pharmacognosy, Standardization.

INTRODUCTION

Curcuma zedoaria Rosc. (Zingeberaceae), commonly known as '*yellow zedoary*' is aromatic, pungent, bitter and useful in flatulent colic and debility of the digestive organs and also used as an ingredient in bitter tincture of zedoary and anti-periodic pills, a paste of rhizome is useful externally for cuts, wounds, itching and in sprains.[1-4]

Rhizomes are employed in Asian and many other countries, including Brazil, for the treatment of several ailments, such as cervical cancer[5,6], hepatitis, inflammations[7] and dolorous processes.[8] Several studies have confirmed and also extended most of the mentioned popular uses of this plant. In this context, several workers were demonstrated its antifungal[9], antiulcer[10], antimutagenic[11], hepatoprotective[12] and cytotoxic[6] properties. It is well-documented that its main active principles are terpenoids, especially sesquiterpenoids[5,6,13,14] which also are produced by cultured cells.[15] *C. zedoaria* is also commonly used in medicine but with high starch content.[16] Phytochemical analysis and analgesic properties of *Curcuma zedoaria* grown in Brazil has also been done.[17]

Traditionally, the dried rhizome of *C. zedoaria* is selected to make drinks or to be extracted as medicine. It has been reported that the boiling water extracts of *C. zedoaria* had a moderate antimutagenic activity against benzo[α] pyrene.[11] Some hepatoprotective sesquiterpenes have been isolated from the aqueous acetone extracts of *C. zedoaria* rhizome. The major sesquiterpene compounds, including furanodiene, germacrone, curdione, neocurdione, curcumenol, isocurcumenol, aerugidiol, zedoarondiol, curcumenone and curcumin, were found to show potent protective effect on d-galactosamine/lipopolysaccharide-induced liver injury in mice.[18]

Although the drug is fairly important and has good economics but no pharmacognostical work have been done in details. Therefore the present study had been done to document its detailed pharmacognostical information which will be utilized by the industries for the authentication and quality control of this drug.

Address for correspondence:
Ph.: +91 522 2297816;
Fax: +91 522 2207219;
E-mail: pharmacognosy1@rediffmail.com

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MATERIALS AND METHODS

Plant material

The plant material was collected from field near Palghat area of Kerala, India, authenticated and lodged in Institute's

Herbarium [LWG 221248, 1999] and the rhizomes were preserved in 70% ethyl alcohol for histological studies. Microtome sections were cut and stained with safranin and fast green and photographed with Nikon F70X camera.[19]

Physico-chemical and phytochemical assays

Physico-chemical and phytochemical studies has been done from the shade dried powdered material according

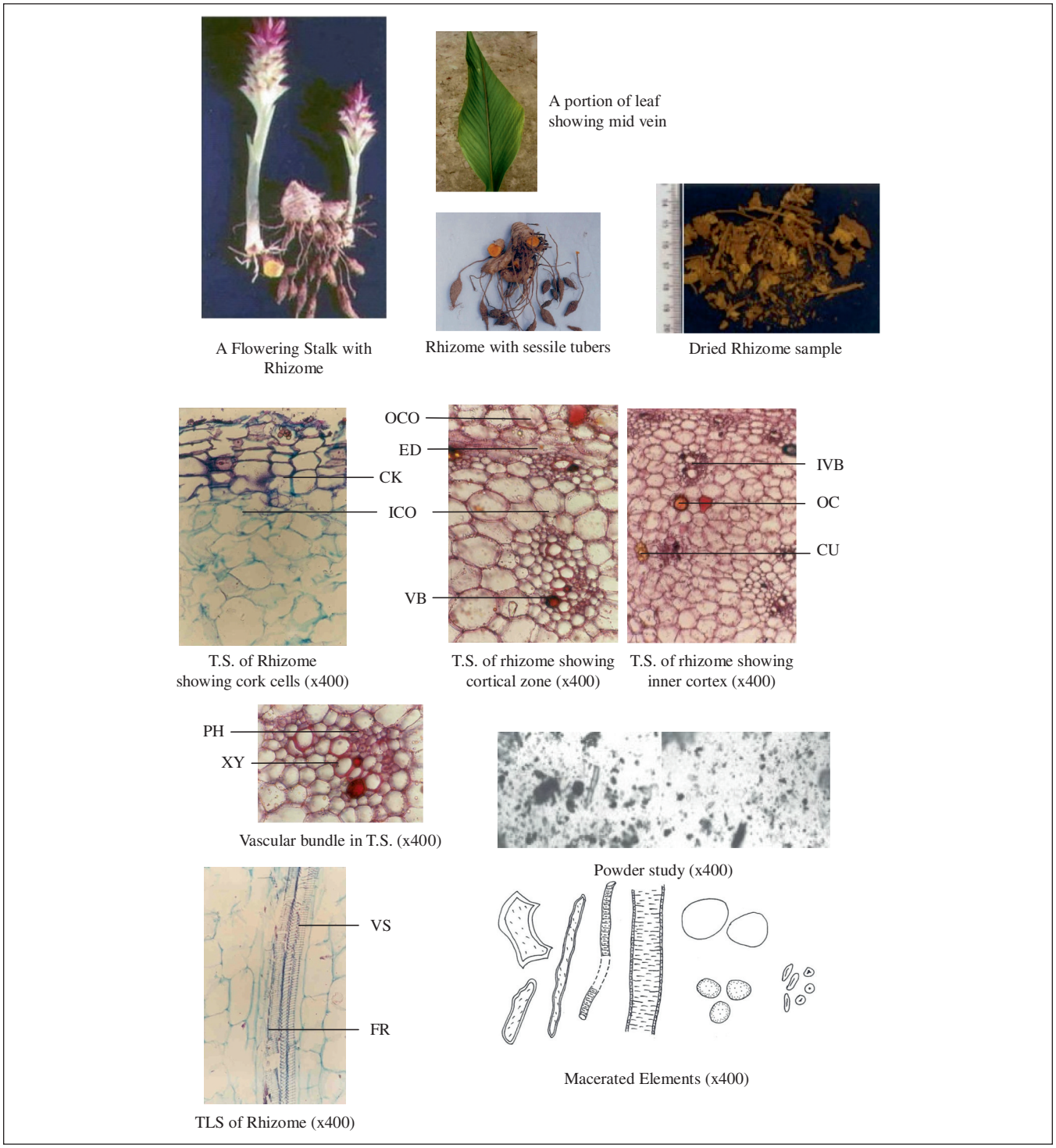

Plate 1: Macro and Microscopic characters of the rhizome of *Curcuma zedoaria* Rosc.

Abbreviations

ICO, Inner cortex; CK, Cork cells; IVB, Inner vascular bundle; OVB, Outer vascular bundle; CO, Cortex; FR, Fibre; ST, Starch; VS, Vessels; XY, Xylem; ED, Endodermis; PR, Pericycle.

to the recommended procedures.[20-22] The behavior of the powdered drug with different chemical reagents was also studied as per methods described.[23,24]

HPTLC studies

The HPTLC analysis was carried out on precoated silica gel 60 F_{254} Merk glass plates of 20 × 10 cm with the help of Camag Linomat- IV applicator and eluted the plate at room temperature in solvent system Chloroform:Ethanol:Acetic acid (95:5:0.1).

RESULTS AND DISCUSSION

Macroscopic characters of the rhizome

The primary rhizome or rootstock is almost conical or top shaped. Attached to the primary rhizome are several sessile

Table 1: Fluorescence powder study of *C. zedoaria* rhizome

S. No.	Treatment	Day light	UV-254 nm	UV-366 nm
1.	Powder (P) as such	Yellowish Brown	Brown	Brown
2.	P. + Nitro-cellulose in amyl acetate	Florescent Yellow	Florescent Yellow	Yellow
3.	P. + N. NaOH in water	Brick Red	Dark Brown	Brown
4.	P. + 1N NaOH + Nitro-cellulose in acetate	Brick Red	Brown with Yellowish Tinge	Black
5.	P. + 1N HCl + Nitro-cellulose in amyl acetate	Florescent Yellow	Florescent Yellow	Yellow
6.	P. + 1N NaOH in Methanol	Brick Red	Brown with Yellowish Tinge	Black
7.	P. + 50% KOH	Brick Red	Brown with Yellowish Tinge	Black
8.	P. 1N HCl	Brown	Light Brown	Black
9.	P. + 50% H_2SO_4	Black	Dark Brown	Black
10.	P. + 50% HNO_3	Brown with Yellowish Tinge	Brown with Greenish Tinge	Brown with Violet Tinge
11.	P. + Conc. HNO_3	Muddy Yellow	Brown with Greenish Tinge	Black
12.	P. + Acetic acid	Light Brown	Light Brown	Black
13.	P. + Conc. H_2SO_4	Black	Black	Black
14.	P. + Iodine water	Black with Greenish Tinge	Black with Greenish Tinge	Black

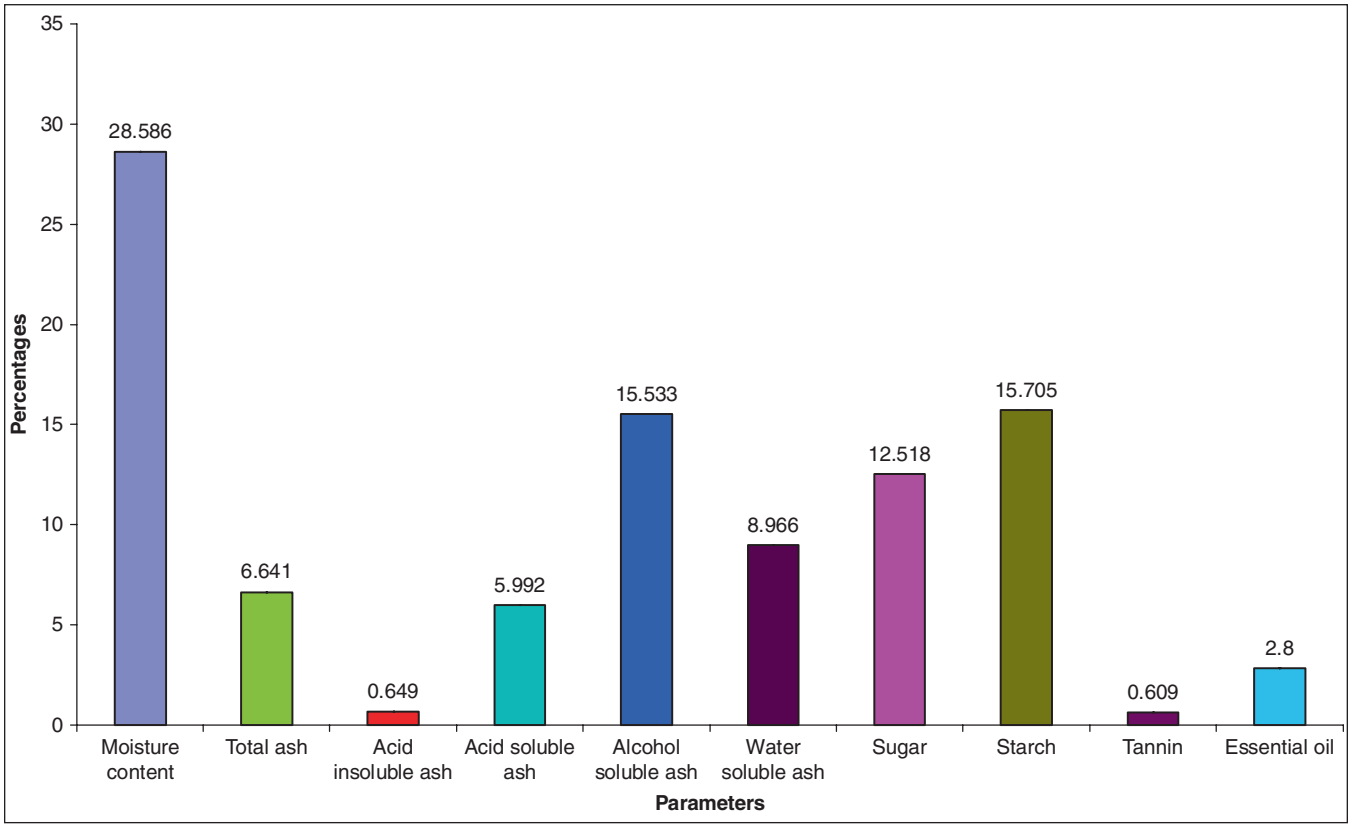

Figure 1: Physicochemical values of *Curcuma zedoaria*

finger shaped lateral branches. The rhizome as well as its branches has an acrid or pungent taste and a distinct aromatic, camphoraceous smell. (Plate 1)

Microscopic characters of the rhizome

Epidermis, the outermost layer composed of rectangular tangentially elongated cells. In older rhizomes the epidermis is replaced by cork composed of 7 to 10 rows of typical rectangular to tangentially elongate thin walled cells. The ground tissue is differentiated into two regions, the outer cortex and the inner cortex by a distinct endodermis. The cortical ground tissue just beneath the cork contain yellowish contents i.e. curcumin. Almost all the parenchymatous cells forming the ground tissue are densely packed with starch grains. The starch grains are simple, comparatively big, flattened, rectangular or ovoid and possess a slight projection at one end. The striations on the grains are numerous. The endodermis is composed of a row of thin walled elongated cells with their radial walls slightly thickened. The cell layer within the endodermis also has tangentially elongated cells but narrower and some of them contain very small oblong starch grains. The cortical bundles as well as those within the stele are similar in structure. Bundles with a single vessel are very rare. Most of the bundles just within the endodermis are small. These contain only 2 to 5 xylem vessels. Each vascular bundle has got a sheath of small sized parenchyma cells, which completely encircle it. The cells forming the sheath, as is the case in the endodermal cells do not contain any starch (Plate 1).

Quantitative microscopy

On maceration, the vessels (744.939 × 15.829 μm) with annular and spiral thickenings are observed. Tracheids with bordered pits measuring 643.453 × 15.123μm are also clearly discernable (Plate 1).

Study powdered rhizome

Powder light yellow, sweet, strong pungent, aromatic odour; shows fragments of storied cork, xylem vessels with reticulate thickenings, lignified xylem fibres, oil cells, patches of parenchymatous cells filled with starch grains which are oval-ellipsoidal, sometimes polygonal in shape, 10 to 60 μm, simple, hilum circular or a 2 to 5 rayed cleft, lamellae distinct and concentric (Plate 1).

The behavior of the powdered drug with different chemical reagents has been shown in the Table 1.

From the above studies rhizome can easily be differentiated on the basis of organoleptic characters for example the odour and taste of rhizome is quite characteristic and is

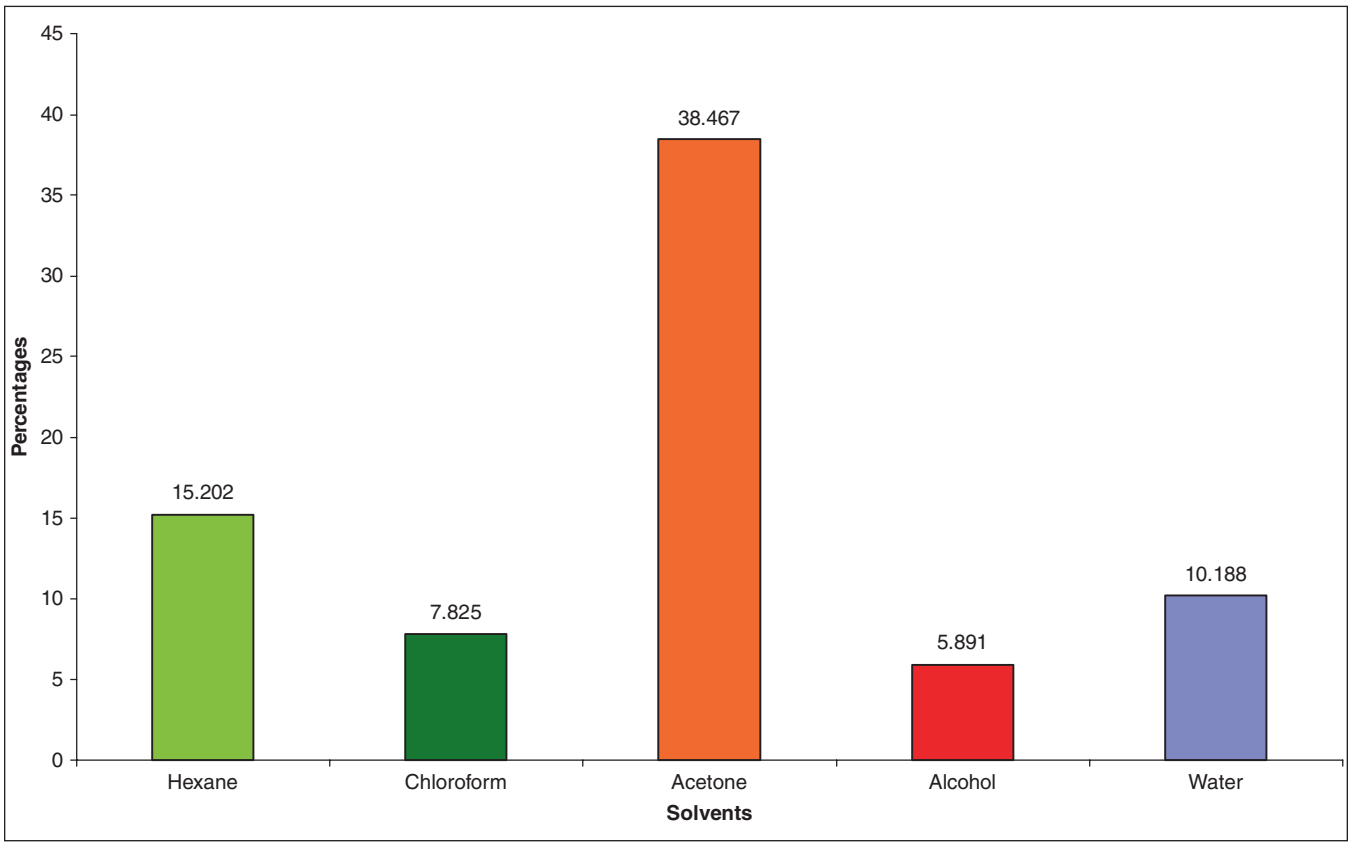

Figure 2: Successive soxhlet values of *Curcuma zedoaria*

Table 2: Phytochemical screening of different extracts of *C. zedoaria* rhizome

Extractive	Triterpe-noids & steroids	Saponins	Flavanoids	Tannins	Reducing sugars	Resins	Glycosides	Alkaloids
Hexane	+	–	–	–	–	–	–	–
Chloroform	–	–	–	–	–	–	–	–
Acetone	+	–	–	–	–	–	–	–
Alcohol	–	–	–	–	–	+	–	+
Water	–	+	–	–	–	–	–	–

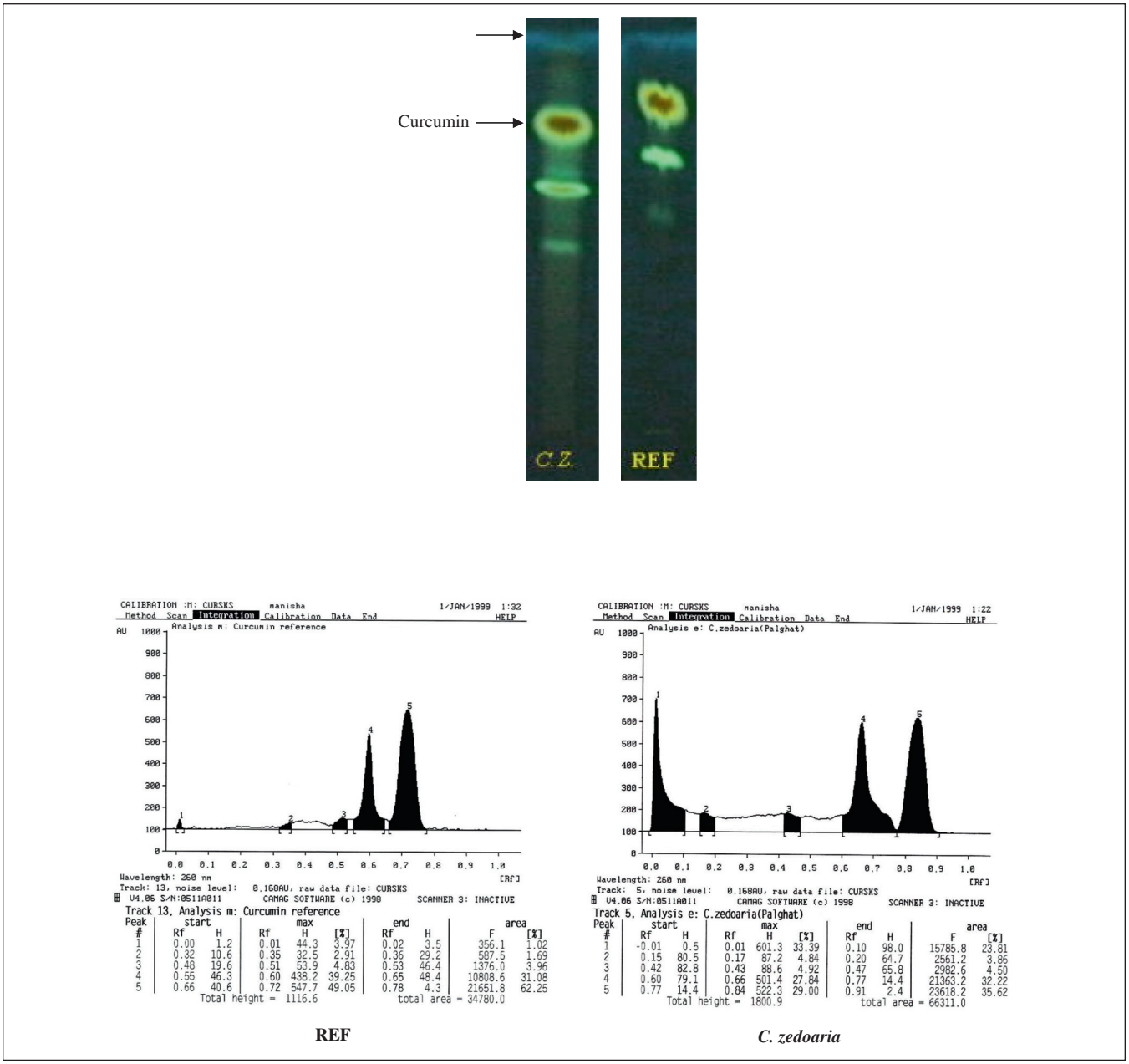

Plate 2: HPTLC Profile of *Curcuma zedoaria* Rosc. and reference sample (Under UV- 366)

1. Reference sample of Curcumin
2. HPTLC profile of *Curcuma zedoaria* Rosc.Rhizome

Abbreviations

REF, curcumin reference; CA, Curcuma zedoaria *Rosc.*

aromatic with pungent taste. On microscopical examination rod shaped starch grains and fibres are observed in the rhizome. Similarly numbers of curcumin containing cells which are yellow in colour are also high in the rhizome.

Physico-chemical studies

Physicochemical values *viz.* percentage of moisture, total ash, acid insoluble ash, alcohol and water-soluble extractives are observed. The total ash and acid insoluble ash, which are considered to be an important and useful parameter for detecting the presence of inorganic substances like silicate ion, it was found 6.64% and 0.64% respectively. Similarly the alcohol and water-soluble extractives, which are indicators of the total solvent soluble components, are 15.53% and 18.96% respectively. Likewise the essential oil, which is an important parameter for identification and authentication it was found to be 2.8% (Figure 1).

Successive Soxhlet extraction from non-polar to polar solvents *viz.* hexane, chloroform, acetone, alcohol and water were also carried out. It is interesting to note that *C. zedoaria* rhizome possessed an exceptionally high amount of acetone extractives i.e. 38.467%, which may be due to the higher percentage of curcumin which is purely soluble in acetone this is also comparable to amount of curcumin in *C. longa* (Figure 2). The preliminary phytochemical screening of different successive extractives is recorded in Table 2.

HPTLC studies

A densitometric HPTLC analysis was also performed for the development of characteristic fingerprint profile, which may be used as markers for quality evaluation and standardization of the drug. The HPTLC analysis was carried out on precoated silica gel 60 F_{254} Merk glass plates of 20 × 10 cm with the help of Camag Linomat- IV applicator and eluted the plate at room temperature in solvent system Chloroform:Ethanol:Acetic acid (95:5:0.1). The bands in the sample are obtained at R_fs 0.17, 0.43, 0.66, and 0.84, which can be used as identifying markers.

The Curcumin was identified at R_f 0.84. (Plate 2)

Heavy metal studies

The various heavy metals viz. Pb, Cd, Co, Mn, Cu, Zn and Hg concentrations was also estimated in the samples and all the metals are found within the permissible limits as prescribed by the WHO. (Figure 3)

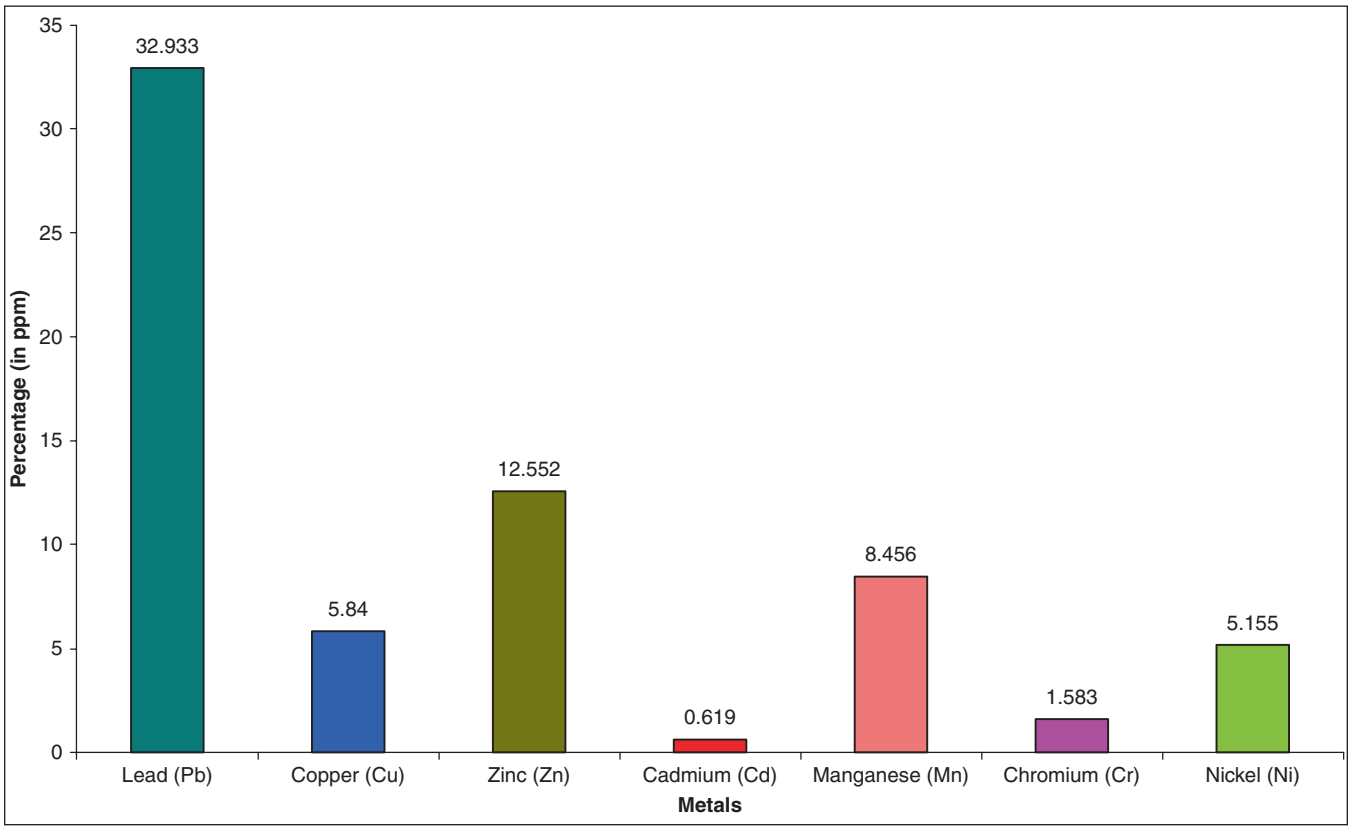

Figure 3: Heavy Metal studies in *Curcuma zedoaria* rhizome

CONCLUSIONS

Thus on the basis of aforesaid studies, it can be concluded that the above parameters are very useful for the identification of the species which may be useful to pharmaceutical industries for the quality control of the commercial samples.

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Antidiabetic activity of fruit pulp of *Feronia elephantum* Corr.

Anurag Mishra*, Ganesh Prasad Garg

Faculty of Pharmacy, Babu Banarasi Das National Institute of Technology & Management, Faizabad Road, Lucknow 227 105 (U.P.) India

ABSTRACT

Feronia elephantum Corr. Commonly known as Bela, Billin, Kath, is a commonly known herb in Indian system of medicine to treat various disorders including diabetes mellitus without any scientific evidences. Therefore this study was designed to investigate *in vivo* hypoglycemic and antidiabetic potential of methanolic extract of fruit pulp of *Feronia elephantum* Corr. in glucose loaded animals and alloxan induced diabetic animals. In both the models *Feronia elephantum* Corr. reduce the blood glucose level when compared to diabetic control group and exert a significant hypoglycemic and antidiabetic activity. However the potency of the herb was less than that of standard drug metformin. *Feronia elephantum* Corr. methanolic extract also reduced the rate of body weight loss in normal and alloxan induced diabetic animals. The results of this study revealed the presence of a significant antidiabetic potential of methanolic extract of *Feronia elephantum* Corr. in alloxan induced diabetic rats. On the basis of this further research work is needed to investigate exact mechanism of action and also to isolate the active constituent/s responsible for the activity.

Key words: Diabetes mellitus, Glucose, Metformin, *Feronia,* Alloxan

INTRODUCTION

Diabetes mellitus (DM) currently is a major health problem for the people of the world and is a chronic metabolic disorder resulting from a variable interaction of hereditary and environmental factors and is characterized by abnormal insulin secretion or insulin recaptor or post receptor events affecting metabolism involving carbohydrates, proteins and fats in addition to damaging liver, kidney and β cells of pancreas.[1] The number of people suffering from the disease worldwide is increasing at an alarming rate with a projected 366 million peoples likely to be diabetic by the year 2030 as against 191 million estimated in 2000.[2] From literature review it has been revealed that 15 - 20% of diabetic patients are suffering from insulin-dependent diabetes mellitus (IDDM) or type-I.[3] In Type-I diabetes mellitus, there is completely destruction of pancreatic β cells and patient is unable to release insulin for maintaining the blood glucose.

Address for correspondence:
Associate Professor, Faculty of Pharmacy,
Babu Banarasi Das National Institute of Technology & Management, Dr. Akhilesh Das Nagar, Faizabad Road, Lucknow 227 105 (India). Mobile No.: +91-9335288099
E. mail: anupriya0522@yahoo.co.in

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In Type-II diabetes mellitus pancreatic β cells partially destruct and/or formation of such proteins opposing the insulin action. The IDDM is noted both in adult and child hood. It is characterized by elevation of both fasting and post-prandial blood sugar levels. Chronic hyperglycemia during diabetes causes glycation of body proteins that in turn leads to secondary complications affecting eyes, kidneys, nerves and arteries.[4] These may be delayed, lessened or prevented by maintaining blood glucose values close to normal in modern medicine; no satisfactory effective therapy is still available to cure the diabetes mellitus. Though insulin therapy is also used for the management of diabetes mellitus, but there are several drawbacks like insulin resistance[5], anorexia, nausea, brain atrophy and fatty liver after chronic treatment. Besides the use of insulin for the treatment of insulin dependent diabetes mellitus (IDDM), other approaches for the control of hyperglycemia include the use of amylin analogues which regulate gastric emptying and inhibitors of intestinal alpha glucosidases like acarbose, miglitol and voglibiose which delay postprandial hyperglycemia. Sulphonylureas, the most widely used class of drugs act by closure of ATP dependent channel. Metformin, a biguanide oral antidiabetic limits intestinal glucose absorption. These drugs have certain effects like causing hypoglycemia at higher doses, liver problems, lactic acidosis and diarrhea. It is apparent that due to the side effects of the currently used drugs, there is a need for a

safe agent with minimal adverse effects, which can be taken for long durations. Though biguanides and sulfonylureas are valuable in treatment of diabetes mellitus, their use is restricted by their limited action, pharmacokinetic properties, secondary failure rates and accompanying side effects.[6] Moreover, these therapies only partially compensate for metabolic derangements seen in diabetics and do not necessarily correct the fundamental biochemical lesion.[7]

Recently, there has been increasing interest in the use of medicinal plants. The use of medicinal plants in modern medicine suffers from the fact that though hundreds of plants are used in the world to prevent or to cure diseases, scientific evidence in terms of modern medicine is lacking in most cases. However today it is necessary to provide scientific proof as whether to justify the use of plant or its active principles.[8]

Feronia elephantum (Corr.) (common names: Bela, Billin, Kath, Kavitha), belongs to family Rutaceae, is a native of Indian subcontinent. The fruit pulp of the plant has been reported in traditional medicine as a curative for various ailments such as diarrheoa, pruritis, impotence, dysentery, heart disease, vomiting, and anorexia, and has also been used for the treatment of asthma and tumours, and as a liver tonic.[9] A decoction (Kadha) administered orally before breakfast has been advocated by local traditional medical practitioners as a tonic purpose.[10] The fruit pulp of *Feronia elephantum* (Corr.) contains flavonoids, phytosterols, tannins, carbohydrates, triterpenoids and amino acids as its chemical constituents.[11]

The gum of the plant are widely used as a curative for diabetes mellitus in Indian system of medicine and also used as a folklore remedy to control the blood glucose level.[10] Hence this study was undertaken to investigate the effect of methanolic extract of *Feronia elephantum* Corr. fruits pulp in normal and alloxanised diabetic rats.

MATERIALS AND METHODS

Plant material

The fruit pulps of *Feronia elephantum* Corr. were collected from the local market of Varanasi and were authenticated by Division of taxonomy, National Botanical Research Institute (NBRI), Lucknow and a voucher specimen no. NBRI/CIF/108/2010 was deposited in national herbarium of NBRI, Lucknow for future reference.

Extract preparation

The dried fruit pulp of the plant (250 g) was comminuted to powder passing through a 60 mesh and then extracted with 95 % methanol using a Soxhlet apparatus. The extract was filtered through cotton wool plug and dried in vacuum on a rotary evaporator (buchi type) at 40-50°C. Complete dryness was achieved in a calcium chloride desiccator and the dry extract was used for all experimental studies.

Phytochemical screening

The preliminary phytochemical screening of the crude methanolic extract of fruit pulp of *Feronia elephantum* Corr. was carried out in order to ascertain the presence of its constituents utilizing standard conventional protocols.[12-14] Phytochemical screening using thin layer chromatography (TLC) was conducted for extract that showed a potential antidiabetic activity against alloxan induced rat diabetes model. Methnolic extract was applied 1 cm above from the base of the TLC plates (0.25 mm, Macherey-Nagel, Germany). Developmet was done using Toluene: methanol: diethyl amine (60:40:5) and Chloroform: methanol: Glacial Acetic Acid (9:1:1) as solvent system specific for various flavonoids and terpenoids respectively. Development of the chromatograms were carried out using anisaldehyde-sulphuric acid spraying reagent and then plates were heated at 105°C in a hot air oven till the spots were developed.[15]

Animals

Healthy male albino Wister rats each weighing 150-200 g were used for study. The rats were housed in polypropylene cages in animal house of BBDNITM, Lucknow and maintained under standard conditions (12 h light and dark cycles, 25 ± 3°C and 55-60% relative humidity). The animals were fed with a standard diet and water *ad libitum*. The study was performed as per the guidelines of IAEC (Institutional Animal Ethics Committee) and Committee for purpose of control and supervision of Experiments on Animals (CPCSEA) and was approved by approval no. BBDNITM/IAEC/01/2010.

Acute toxicity study

The 'Up and Down' or 'Staircase' method was adopted for this evaluation. The extract was administered orally in a dose range of 200-5000 mg/kg body weight to ten groups of mice ($n = 6$). Two mice were orally dosed with 250 mg/kg and observed for a period of 24 h for mortality. In this approach, subsequent doses were then increased by a factor 1.5 if the dose was tolerated, or, decreased by a factor of 0.7 if it was lethal. The maximum non-lethal and minimum lethal doses were determined using 6 mice. Those mice which received doses above 5000 mg/kg body weight exhibited ptosis (drooping of upper eyelid) and were observed to be lethargic. Once the approximate LD_{50}, or the range between the maximum non-lethal and minimum lethal dose was found, a final and more reliable LD_{50} assay was performed using at least 3 or 4 dose levels within this range with a larger number of animals in each group. In addition, the source of animal, sex, age, body weight, and presence or absences of any immediate reaction were also recorded as per CPCSEA protocol.[16] The duration of the toxicity test was two weeks.

Induction of diabetes mellitus

Twenty four male Albino Wistar rats weighing 150-200 grams were used for the study of the effects of *Feronia elephantum* extract on the blood glucose levels of the animals. The animals were fed on commercial feeds and were given water *ad libitum*. The animals were fasted from feeds for 12 hours before the commencement of each experiment, but were allowed water *ad libitum*. The rats were injected with alloxan mono hydrate suspended in water at a dose of 120 *mg*/kg body weight intraperitonealy. They were kept for the next 24 hours on 5% glucose solution bottles in their cages to prevent hypoglycemia. After a period of three days the rats with a blood glucose levels greater than 150 *mg*/dl were considered diabetic and used for current research work.[17]

Experimental procedure for ogtt and antidiabetic activity

The animals for oral glucose tolerance test were randomly assigned into four groups of six rats in each group ($n = 6$) each as follows, namely

Group 1 - Normal undiseased animals who only received normal saline (Normal Control).

Group 2 - Diseased animals who received glucose (1.75 *g*/kg p. o.)

Group 3 - Diseased animals who first received metformin 100 *mg*/kg and then glucose (2 hrs. later) 1.75 *g*/kg p. o.

Group 4 - Diseased animals treated methanol extract of *Feronia elephantum* Corr. 500 *mg*/kg

Oral glucose tolerance tests were performed on 16 hr fasted Wister rats using 1.75 *g* glucose per kg body weight fed orally (dissolved in water for injection) through a canula fitted needle attached to syringe. Just after glucose fed single dose of plant sample (methanolic extract of fruit pulp of *Feronia elephant* Corr.) was also fed to study the effect of the same on GTT. The control group were fed equal amount of vehicle solutions orally.[17]

In all the groups, blood was collected from the animal's tail vein at 0, 30, 60, 90 minutes after glucose feeding. Simultaneously, blood glucose was measured by trinder's glucose oxidase method using spectrophotometer.

Table 1: Effect of extract on oral glucose tolerance test in normal rats

Groups	Dose	Blood glucose level (*mg*/dl) (mean ± sem)			
		0 minute	30 min	60 min	90 min
Group I (normal control)	Normal saline	103.83 + 2.49	105.65 ± 2.94	104 ± 3.36	104.59 ± 2.61
Group II (disease control)	1.75 g/kg	105.05 ± 1.54	201.66 ± 2.40	267.83 ± 1.60	198.67 ± 1.74
Group III (standard)	100 *mg*/kg	104.30 ± 1.52**	115.95 ± 1.50**	115.70 ± 3.99**	101.5 ± 1.23**
Group IV (Test)	500 *mg*/kg	107.38 ± 1.91**	130.00 ± 2.51**	134.46 ± 2.74**	123.0 + 2.08**

Group I - normal control; **Group II** - Diseased control; **Group III** - Diseased animals treated with standard drug Metformin; **Group IV** - Diseased animals treated methanol extract of *Feronia elephatum* Corr. 500 *mg*/kg
Values are expressed in Mean ± SEM ($n = 6$), $P^{**} < 0.05$ when compared to Group II.

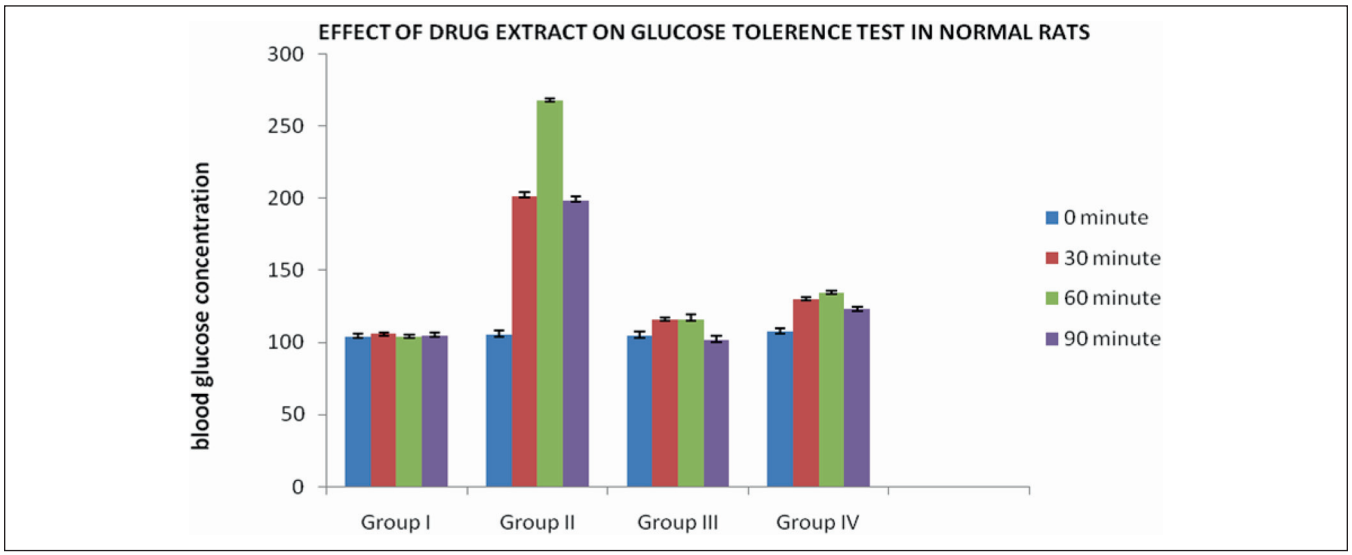

Figure 1: Effect of drug extract on glucose tolerance test in normal rats

The animals for Alloxan-induced diabetic study were randomly assigned into four groups (1-4) of six rats ($n = 6$) each as follows, namely

Group 1 - Received only normal saline orally

Group 2 - Diseased animals who received only alloxan monohydrate (120 *mg*/kg i. p.) Diseased control

Group 3 - Diseased animals who first received alloxan monohydrate (120 *mg*/kg body weight) once and then metformin (standard drug) 100 *mg*/kg body weight for 21 days.

Group 4 - Diseased animals treated methanolic extract of *Feronia elephantum* Corr. 500 *mg*/kg body weight for 21 days.[17]

Determination of blood glucose levels

Blood samples were collected by cutting the tail-tip of the rats, for blood glucose determination at intervals of 1st, 7th, 14th and 21st day. Determination of the blood glucose level was done by the glucose-oxidase principle using the ONE TOUCH Basic (Lifescan, Milpitas, CA) instrument and results were reported as *mg*/dl.

Statistical analysis

Blood glucose levels were expressed in *mg*/dl as mean ± SEM (standard error of mean). The data were statistically analyzed using one way ANOVA by Dunnett test. The comparision was made reference group and test group versus disease control group. The values of $P < 0.05$ and $P < 0.01$ were considered as significant.

RESULTS AND DISCUSSION

Phytochemical analysis

Freshly prepared extract were subjected to preliminary phytochemical screening test for various constituents. This revealed the presence of tannins, coumarins, flavonoids and essential oils (terpenoids) mainly. Further thin layer chromatography studies confirmed the presence of these phytoconstituents in the extract.

Pharmacological screening

Determination of acute toxicity and ld_{50} values

After using various doses level in various groups the toxicological data and LD_{50} values was determined for extract.

Table 2: Effect of extract on blood glucose level in alloxan Induced diabetic rats

Group	Dose	Blood glucose level (*mg*/dl) (mean ± SEM)			
		1st day	7th day	14th day	21st day
Group I (normal control)	--------	97.62 ± 2.72	98.560 ± 1.77	91.920 ± 02.18	87.340 ± 02.18
Group II (disease control)	120 *mg*/kg	194 ± 11.09	210.1 ± 7.48	235.2 ± 4.52	240.3 ± 9.82
Group III (standard)	100 *mg*/kg	167.54 ± 3.67*	165.85 ± 2.54*	132.20 ± 2.47*	115.25 ± 2.23*
Group IV (Test)	500 *mg*/kg	180.30 ± 5.2*	175.70 ± 1.7*	145.65 ± 5.76*	130.9 ± 6.70*

Group I - normal control; **Group II** - Diseased control **Group III** - Diseased animals treated with standard drug Metformin; **Group IV** - Diseased animals treated methanol extract of *Feronia elephatum* Corr. 500 *mg*/kg
Values are expressed in Mean ± SEM ($n = 6$), $P^* < 0.01$ when compared to Group II

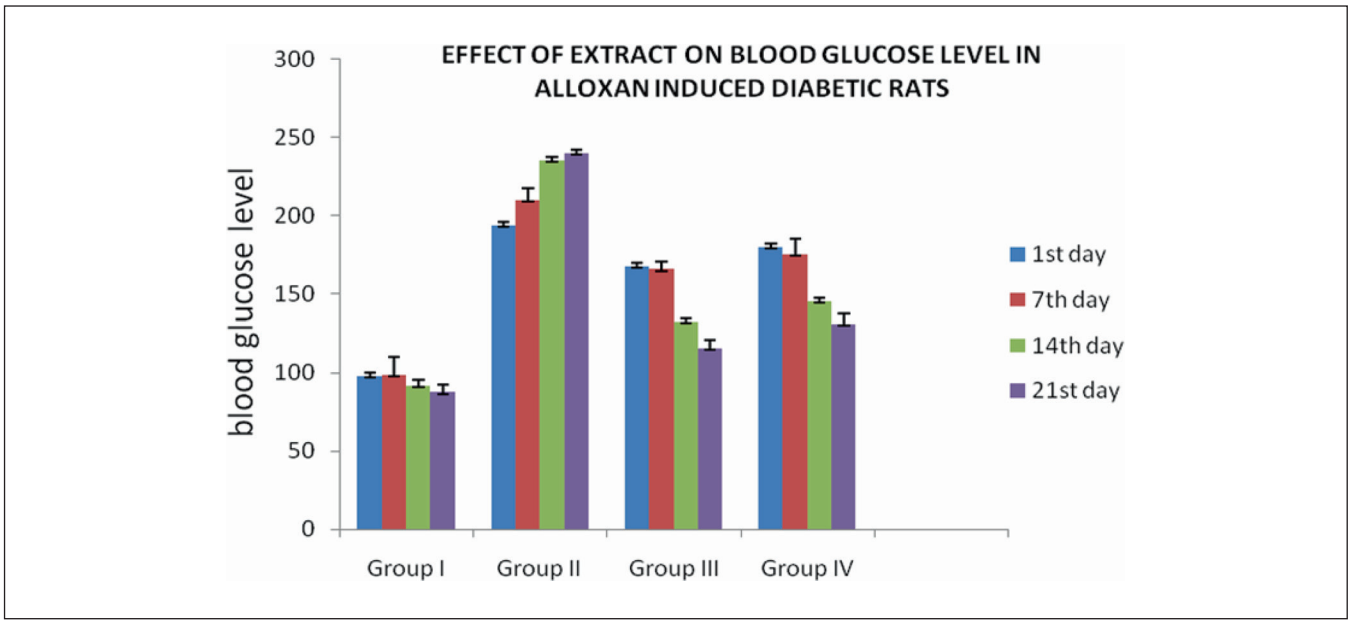

Figure 2: Effect of extract on blood glucose level in alloxan induced diabetic rats

No mortality was seen upto doses as high as 5 *g*/kg by staircase method. So a dose well below the possibly toxic (approximately 1/10th) of 500 mg/kg was taken.

DISCUSSION AND CONCLUSION

This study firstly evaluated the hypoglycemic effect of *Feronia elephantum* Corr. in glucose induced hyperglycemia and alloxan induced diabetic rats. It was found that pretreatment of *Feronia elephantum* methanolic extract in normal rats at a dose level of 500 *mg*/kg body weight caused a partial prevention of hyperglycemia induced by glucose (1.75 *g*/kg body weight). Methanol extract significantly reduced the blood glucose level after 90 minute of administration when compared to control group. In alloxan induced diabetes rat *Feronia elephantum* methanolic extract showed a significant decrease in blood glucose level when treated for 21 days at a dose level of 500 *mg*/kg body weight.

It was also observed that *Feronia elephantum* Corr. fruit pulp methanolic extract when administered to alloxan induced diabetic rats, the weight loss was reversed and the animal returned to near normal when compared to disease control group. The ability of the methanolic extract of fruit pulp to protect body weight loss seems to be due to its ability to reduce hyperglycemia.

The possible mechanism by which fruits bring about a decrease in blood sugar level may be by potentiation of the insulin effect of plasma by increasing either the pancreatic secretion of insulin from β cells of the islets of Langerhans or its release from the bound form. A number of other plants have been reported to exert hypoglycemic activity through insulin release-stimulatory effect.

To our knowledge, this is the first study in which methanolic extract of fruits pulp of *Feronia elephantum* Corr. was proved *in vivo* a potent hypoglycemic/anti-hyperglycemic agent and this information can be useful for the management of Type-I as well as Type-II diabetes mellitus. On the basis of this study further research works are needed to understand the exact mechanism of action of hypoglycemia produced by drug and to isolate the moieties responsible for the activity.

Table 3: Body weight of the animal during the experiment in alloxan induced diabetic rats

Groups	Initial Body Weight	Final Body Weight
Group I (normal control)	162.47 ± 3.98	179.67 ± 3.42
Group II (disease control)	165.24 ± 6.74	125.87 ± 6.67
Group III (standard)	164.34 ± 6.45	158.23 ± 4.98
Group IV (Test)	170.54 ± 7.60	142.21 ± 3.77

Group I - normal control; **Group II** - Diseased control; **Group III** - Diseased animals treated with standard drug Metformin **Group IV** - Diseased animals treated methanol extract of *Feronia elephatum* Corr. 500 mg/kg
Values are expressed in Mean ± SEM (n = 6),

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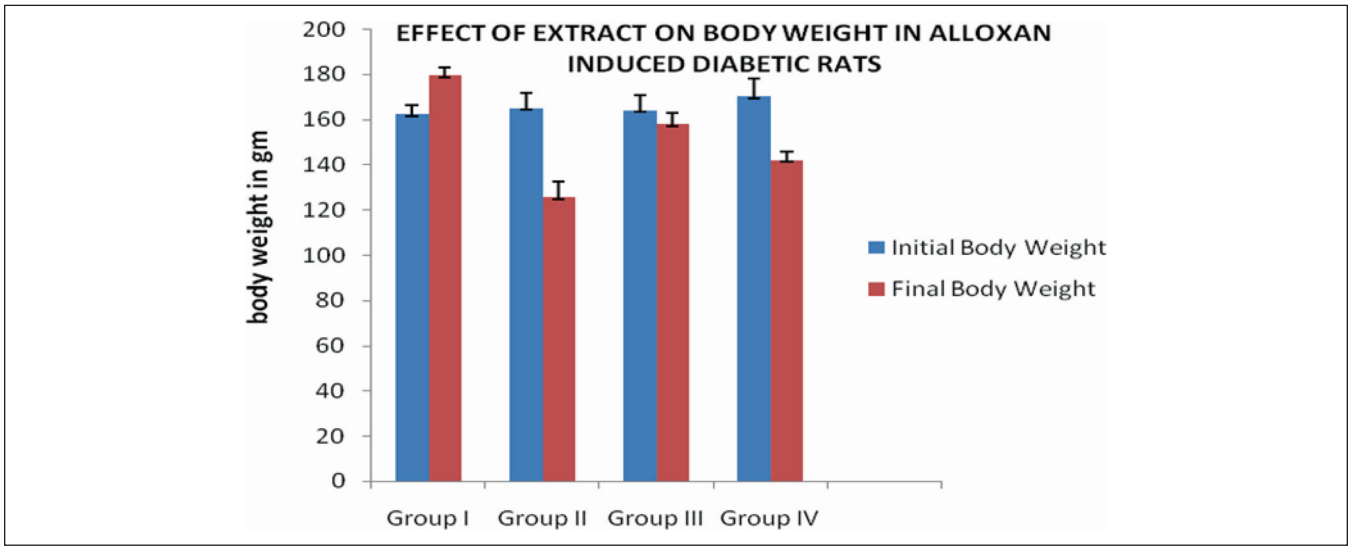

Figure 3: Effect of extract on body weight in alloxan induced diabetic rats

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Inhibitory activities of *Ficus benghalensis* bark against carbohydrate hydrolyzing enzymes - An *in vitro* study

Faiyaz Ahmed, Shailesh Chavan[1], Satish A, Punith Kumar R

Nutra Bio Innovations, Vijaynagar, Mysore, India. [1]Biotest Pharmaceuticals, Boca Raton, Florida, USA

ABSTRACT

The present study evaluated the effect of *Ficus benghalensis* Linn. (Moraceae) stem bark on porcine pancreatic α-amylase, rat intestinal α-glucosidase and sucrase. Further, the effect of heat treatment was also studied. Both untreated and heat-treated samples inhibited α-amylase to a significant extent. Further, the aqueous extracts prepared form untreated and heat treated samples exhibited significant inhibition ($p \leq 0.05$) of α-glucosidase and sucrase in a dose dependent manner. Heat treatment of the sample increased α-glucosidase and sucrase inhibitory activities at lower concentrations, however no statistical differences were observed at higher concentrations. Consequently, the untreated extracts showed IC_{50} values of 158 and 193 µg mL^{-1} for α-glucosidase and sucrase respectively while, the heat-treated extracts showed the IC_{50} values 77 and 141 µg mL^{-1} respectively. Further, a significant correlation ($p \leq 0.05$; $r = 0.698$) was observed between α-glucosidase and sucrase inhibitory activities of both untreated and heat-treated extracts. The results clearly demonstrate that inhibition of carbohydrate hydrolyzing enzymes is one of the mechanism through which *F. benghalensis* bark exerts its hypoglycemic effect *in vivo*.

Key words: *Ficus bengalensis,* α-amylase, α-glucosidase, sucrase, diabetes

INTRODUCTION

Diabetes mellitus is a metabolic disorder of multiple aetiology characterized by chronic hyperglycemia with disturbance of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both.[1] Control of postprandial plasma glucose rise is critical in the early treatment of diabetes mellitus[2] as studies indicate that postprandial hyperglycemia induces non-enzymatic glycosylation of various proteins, resulting in the development of chronic complications such as micro and macro vascular diseases.[3] Postprandial glucose rise can be controlled by reducing/delaying the absorption of glucose through the inhibition of the carbohydrate hydrolyzing enzymes such as α-amylase, α-glucosidase, β-glucosidase and sucrase, in the digestive tract. Inhibitors of these enzymes delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the post-prandial hyperglycemia.[4] A number of alpha glucosidase inhibitors have been recently developed from the natural sources,[5-6] of which acarbose, miglitol and voglibose are in clinical use.[7]

Ficus benghalensis Linn (Moraceae) commonly known as banyan tree in English and bargad in Hindi is named after the Hindu traders, called Banyans, who favored the tree.[8] Different parts of *F. benghalensis* tree have been found to possess medicinal properties; leaves are good for ulcers, aerial roots are useful in gonorrhea, seeds and fruits are cooling and tonic.[9] The bark is astringent and is useful in the treatment of dysentery, diarrhea and diabetes. Stem bark is used as antihelminthic.[10] *F. benghalensis* is one of the most widely explored medicinal plants for the antidiabetic activity wherein, the antidiabetic potential of various parts of *F. benghalensis,* particularly of the bark has been evaluated in experimental diabetes using alloxan/ streptozotocin as diabetogenic compounds in animal models. It is one medicinal plant whose active components such as perlargonidin derivatives, leucopelargonin derivatives and α-amyrin acetate have been extensively evaluated for the antidiabetic activity *in vivo*.

In the present investigation *Ficus benghalensis* stem bark, a proven hypoglycemic agent,[11-12] being used by various cultures across India for the treatment of diabetes was studied for its ability to inhibit the carbohydrate hydrolyzing

Address for correspondence:
E-mail: fayaz_ahmed09@yahoo.co.in

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enzymes using *in vitro* model systems. Further, the effect of heat treatment on the enzyme inhibitory activities was also studied.

MATERIALS AND METHODS

Chemicals and reagents

Porcine pancreatic α-amylase (23 u/mg solid) was purchased from Sigma Aldrich, India. p-nitrophenyl-α-D-glucopyranoside and 3,5-dinitrosalysilic acid were purchased from Sisco Research Laboratory, India. Glucose oxidase peroxidase assay kit was purchased from Agappe Diagnostics, India. All the chemicals and reagents used in the study were of extra pure analytical grade.

Collection of plant material

Ficus bengalensis stem bark was collected form a mature tree in the campus of University of Mysore, India and was identified by Dr. Niranjan, Department of Botany, University of Mysore, India. The bark was cut into small pieces, dried at 50°C overnight, powdered and passed through 60 mesh sieve (BS) and stored in an air tight container at 4°C till further use.

Heat treatment

The bark powder was subjected to heat treatment in a vacuum oven at 100°C for 60 minutes, cooled in a desiccator and used for the preparation of the heat-treated extract (FBH).

Preparation of extracts

Aqueous extracts (Untreated and heat-treated) were prepared by extracting the untreated and heat-treated bark powders with distilled water (1:8 w/v) on a mechanical shaker, for 24 hours, at room temperature.

Assay of α-amylase inhibitory activity

The effect of *F. benghalensis* on α-amylase activity was studied using an enzyme-starch system.[13] *F. benghalensis* powder (1%) was mixed by stirring with 25 mL of 4% potato starch in a beaker; 100 mg of α-amylase was added to the starch solution, stirred vigorously, and incubated at 37°C for 60 minutes. After the incubation period 0.1 M NaOH was added, to terminate enzyme activity. The mixture was centrifuged (3000 xg; 15 minutes) and the glucose content in the supernatant was determined.

Assay of α-glucosidase inhibitory activity

A crude enzyme solution of rat intestinal α-glucosidase and sucrase, prepared according to the method of Dahlqvist,[14] was used to assay the α-glucosidase and sucrase inhibitory activities, according to the method of Honda and Hara.[15] Ten milliliters of enzyme solution and varying concentrations of the aqueous extract (100-500 µg) were incubated together for 10 minutes, at 37°C, and the volume was made up to 210 µL with maleate buffer, pH 6.0. The enzyme reaction was started by adding 200 µl of 2 mM p-nitrophenyl-α-D-glucopyranoside solution and further incubated at 37°C for 30 minutes. The reaction was terminated by treating the mixture in a boiling water bath for five minutes. After the addition of 1.0 ml of 0.1 M disodium hydrogenphosphate solution, the absorption of liberated p-nitrophenol was read at 400 nm.

Assay of sucrase inhibitory activity

The effect of *F. benghalensis* on sucrase activity was assayed according to the method of Honda and Hara.[15] The enzyme solution (10 µL) and varying concentrations of the aqueous extract (100-500 µg) were incubated together for 10 minutes at 37°C, and the volume was made up to 200 µL with maleate buffer (pH 6.0). The enzyme reaction was started by adding 100 µl sucrose solution (60 mM). After 30 minutes, the reaction was terminated by adding 200 µL of 3,5- dinitrosalysilic acid reagent and treating the mixture in a boiling water bath for five minutes. The absorbance of the solution was read at 540 nm.

The percent inhibitory activities were calculated using the following formula:

$$\%\ \textit{inhibition} = \frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100$$

Where, *Abs control* is the absorbance of the control reaction (containing all reagents except the test sample), and the *Abs sample* is the absorbance of the test sample. An untreated enzyme solution was used as the control. All the experiments were carried out in triplicate.

Statistical analysis

The data was analyzed by ANOVA followed by Tukey's multiple comparisons test for significant differences, using SPSS 14.0 computer software. The values were considered significant when $p \leq 0.05$. IC_{50} values were calculated by Boltzmann's dose response analysis using Origin 6.1 software.

RESULTS

Effect of *Ficus benghalensis* on α-amylase activity

The α-amylase inhibitory activity of *F. benghalensis* powder (FBP) was studied using α-amylase-starch model system and the results indicate that untreated FBP inhibited α-amylase to an extent of 50% at the concentration of 1%, while heat-treated FBP inhibited α-amylase by 47% at the same concentration. No statistical difference was observed

between the α-amylase inhibitory activity of untreated and heat-treated FBP.

Effect of *Ficus benghalensis* on α-glucosidase and sucrase activity

The effect of *F. benghalensis* extracts on α-glucosidase activity is shown in Figure 1. Both the untreated and heat treated extracts significantly inhibited ($p \leq 0.01$) α-glucosidase in a dose dependent manner. The inhibitory activities ranged between 35-97% and 55-98% respectively for untreated and heat-treated extracts. The IC_{50} value of heat-treated extract was significantly lower ($p \leq 0.05$) than that of untreated extract. An IC_{50} value of 77 μg mL^{-1} was observed for heat-treated extract while, the IC_{50} value of untreated extract was found to be 158 μg mL^{-1}.

Effect of *Ficus benghalensis extracts* on sucrase activity

The sucrase inhibitory activity ranged between 40-71% and 45-73% for untreated and heat-treated *F. benghalensis* extracts respectively (Figure 2). A dose dependent inhibition of rat intestinal sucrase was observed in both untreated and heat treated *F. benghalensis* extracts and heat treatment did not result in any significant change ($p \leq 0.05$) in the sucrase inhibitory activity of the sample. Consequently, no significant difference ($p \leq 0.05$) was observed between the IC_{50} values of both the extracts. The IC_{50} value for heat-treated extract was 141 ± 22.1 while, the IC_{50} value for untreated extract was 193 ± 21.6.

A significant correlation ($p \leq 0.05$; $r = 0.698$) was observed between α-glucosidase and sucrase inhibitory activities of both untreated and heat treated *F. benghalensis* extracts and the enzyme inhibitory activities of *F. benghalensis* extracts were directly proportional to the sample concentration.

Table 1: IC_{50} values for α-glucosidase, and sucrase activities (μg mL^{-1})

Enzyme	FBU	FBH
α-glucosidase	158[b] ± 4.1	77.1[a] ± 8.7
Sucrase	193[a] ± 21.6	141[a] ± 22.1

*FBU: untreated *Ficus benghalensis* extract, FBH: heat-treated *Ficus benghalensis* extract
**Mean values carrying different superscript letters a & b in rows, differ significantly ($p \leq 0.05$)

DISCUSSION

The present study evaluated the effect of *F. benghalensis* bark on carbohydrate hydrolyzing enzymes viz. α-amylase, α-glucosidase and sucrose since, the development of antidiabetic drugs with complementary mechanisms of action appears essential to achieve good glycemic control by inhibiting in a reversible way the hydrolysis of carbohydrates, to reduce postprandial blood glucose rise in type 2 diabetics.[16]

Several mechanisms have been proposed for the hypoglycemic effect of phytochemicals, such as inhibition of carbohydrate

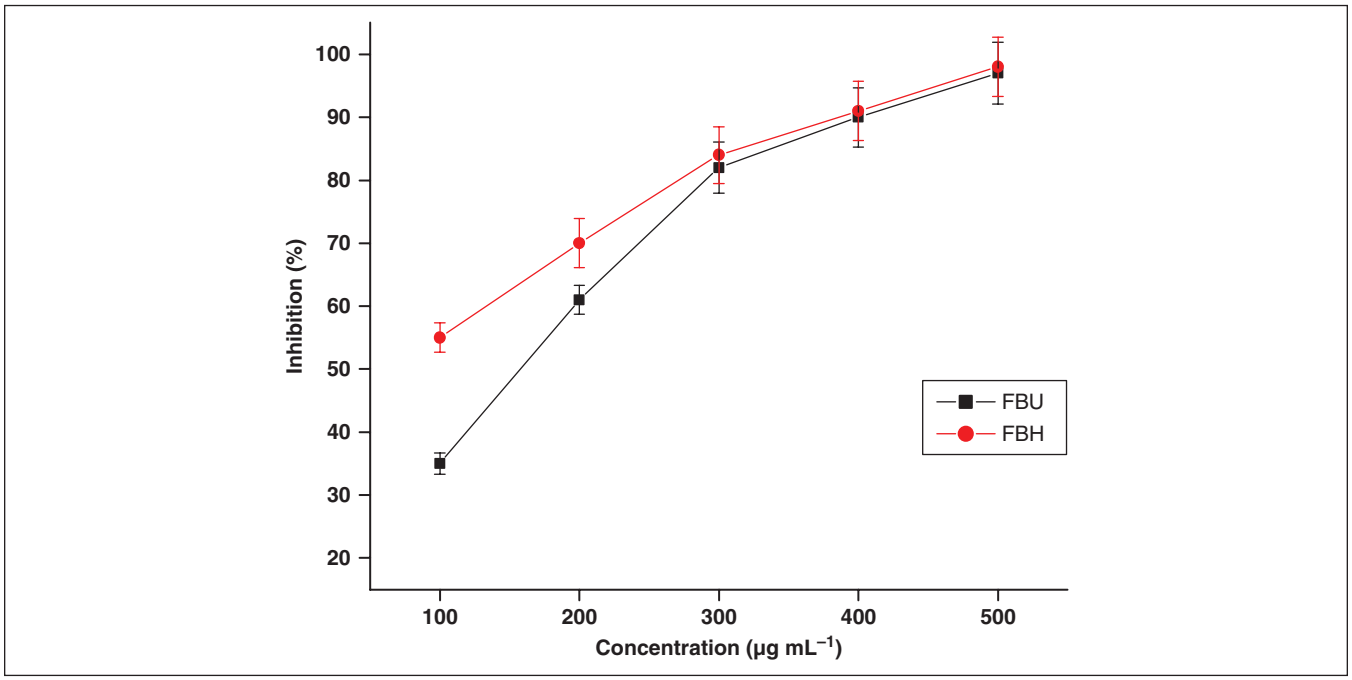

Figure 1: Effect of *Ficus benghalensis* bark extracts on α-glucosidase activity.
FBU: untreated Ficus benghalensis *extract, FBH: heat-treated* Ficus benghalensis *extract*

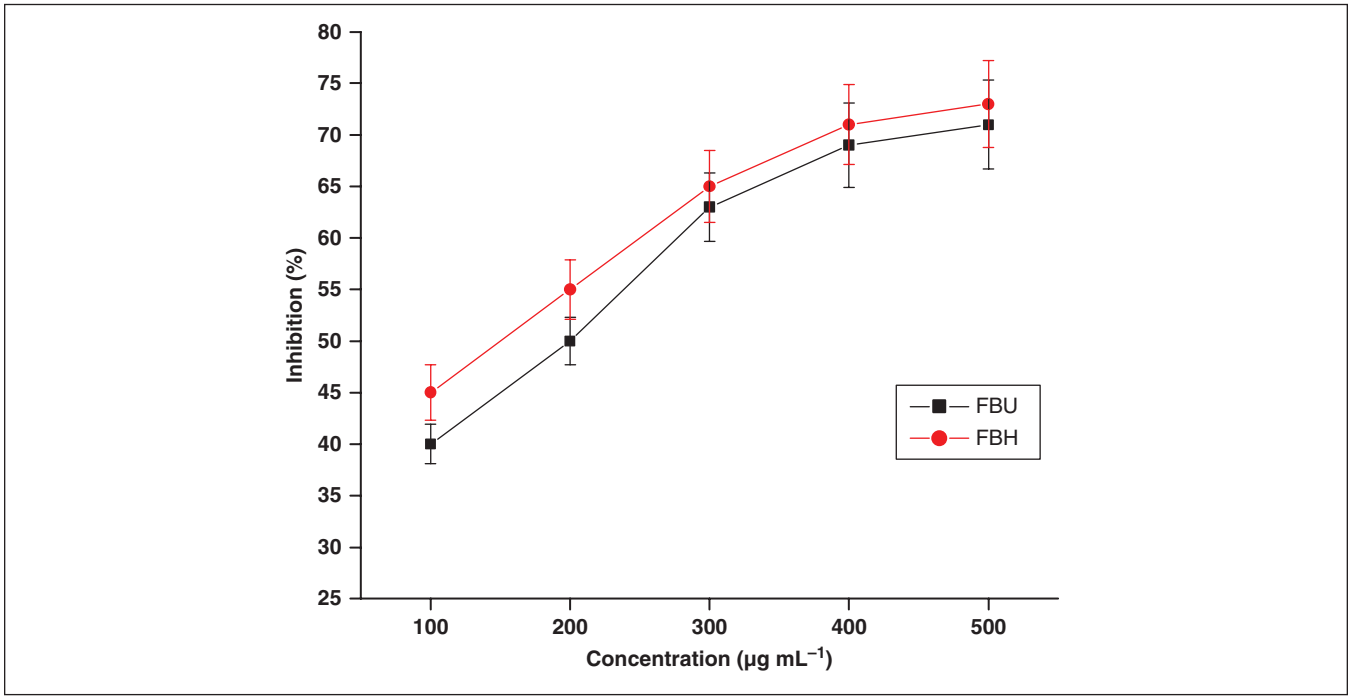

Figure 2: Effect of *Ficus benghalensis* bark extracts on sucrase activity.
FBU: untreated Ficus benghalensis *extract, FBH: heat-treated* Ficus benghalensis *extract*

metabolizing enzymes, manipulation of glucose transporters, β-cell regeneration and enhancing insulin releasing activity and sensitivity.[17] In the present investigation, both untreated and heat treated samples effectively inhibited porcine pancreatic α-amylase which can possibly be attributed to several factors such as fiber concentration, presence of inhibitors on fibers, encapsulation of starch and enzyme by the fibers present in the sample thereby reducing accessibility of starch to the enzyme and direct adsorption of the enzyme on fibers leading to decreased amylase activity.[13]

Glucosidases are crucial in many biological processes, including breakdown of edible carbohydrates.[18] α-glucosidase is one among the number of glucosidases located in the brush-border surface membrane of intestinal cells, and is a key enzyme of carbohydrate digestion.[19] The inhibition of α-glucosidase by *F. benghalensis* bark can be attributed to the presence of flavonoids and phenolic glycosides[20] having potential antioxidant activity, as reports indicate that phenolic enriched extracts of *Solanum melongena* with moderate free radical scavenging linked antioxidant activity had high α-glucosidase inhibitory activity. Inhibition of this enzyme provides a strong biochemical basis for management of type 2 diabetes by controlling glucose absorption.

The increase in the α-glucosidase by heat treatment may be due possible inactivation of the phytoconstituents that hinder/decrease the inhibition of glucosidases by the heat treatment. The main benefits attributable to α-glucosidase inhibitors are reductions in both postprandial glycemic levels and in the total range of postprandial glucose levels.[21]

Rat intestinal sucrase occurs as a complex of sucrase and isomaltase which converts sucrose into glucose.[22] The inhibition of sucrase by *F. benghalensis* bark extracts may also be due to its phenolic compounds. Further, the correlation observed between α-glucosidase and sucrase inhibitory activities of both untreated and heat-treated extracts represents a parallel and effective inhibition of the brush border enzymes in the digestive tract.

CONCLUSION

The findings of the present study emphasizes that, inhibition of carbohydrate hydrolyzing enzymes such as α-amylase, α-glucosidase and sucrase is one of the mechanisms through which *F. benghalensis* bark exerts its hypoglycemic effect *in vivo*. The study also supports the traditional usage of *F. benghalensis* bark as an antidiabetic agent.

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Evaluation of antiulcer activity of various extracts of *Semecarpus anacardium* seeds

Kumar Manoj*, Jangra Meenu[1], Sharma Sunil[2]

*[1]Lecturer, Department of Pharmacy, Saroj Institute of Engg. & Management, Lucknow,
[2]Asst. Prof., Guru Jambheshwar University of Sc. & Tech , Hisar, India

ABSTRACT

Methanolic and aqueous extracts of *Semecarpus anacardium* seeds were investigated for gastric protective activity on ethanol plus pylorus ligation induced ulcer models. A number of biochemical parameters such as gastric volume, pH of gastric content, free acidity and total acidity, dissolved mucous substances such as total protein, hexoses, hexosamine, fucose and sialic acid were estimated in 90% alcoholic precipitate of gastric juice and macroscopically sections were examined on the test and control group animals. The extract at a concentration of 200mg/kg produced a protective effect on ulcer-induced models and was comparable with the standard drug cimetidine. Some of the antioxidant enzyme levels (lipid peroxidation, superoxide dismutase and catalase) were also determined. The present study revealed that the methanolic extract of *Semecarpus anacardium* had ulcer protective activity comparable with standard drug cimetidine.

Key words: *S. anacardium*, ulcer, Ethanol plus pylorus ligation, Biochemical parameters, Methanolic extract

INTRODUCTION

Peptic ulcer disease is a serious gastrointestinal disorder. The formation of peptic ulcers depends on the presence of acid and peptic activity in gastric juice plus a breakdown in mucosal defences. There are two major factors that can disrupt the mucosal resistance to injury: non-steroidal anti-inflammatory drugs (NSAIDs) e.g. aspirin and *Helicobacter pylori* infection.[1] As a matter of fact, many drugs were used to treat this disease but many of them cause adverse effects and recurrent infections frequently occur within a few weeks because of difficulty in eradication of *H. pylori*.[2] This has been rationale for the development of new antiulcer drugs and search for novel molecule. Drugs of plants origin are gaining popularity and investigating for the various disorders including peptic ulcer. The objective of present study was to evaluate the effectiveness of seeds extract in preventing the formation of gastric ulcer experimentally by ethanol-induced gastric damage in rats. Cimetidine was used as reference drug for comparison.

Address for correspondence:
Tel: +91 9208877924
E-mail: meenujngr@yahoo.in;

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Seeds of *Semecarpus anacardium* (Marking nut) are used in Indian traditional medicines (Ayurveda and Sidha) either alone or as an ingredient of many polyherbal formulation for treating various ailments. *S. ancardium* of Anacardiaceae family is a medium sized tree grown in arid parts of tropical and subtropical regions. Ayurveda describes it as a potent drug for neuritis, arthritis, leprosy, helmintic infection and venereal disorders[3,4] But supporting data are lacking. Recently antioxidant,[5,6] anti-inflammatory,[7] anti-cancer,[8,9] antibacterial,[10] anti-rheumatic[11] and anthelminthic[4] activities of its seeds have been reported. A variety of flavonoids such as tetrahydroamentoflavone(THA),[12] jeediflavanone,[13,14] semicarpouflavonone,[15] galluflavonone,[16] nallaflavonone,[17] semecarpeti[18] and anacardioflavonone[19] along with other phenolic compounds such as bhilawanols and anacardic acids[20] have been reported. The present study was undertaken to assess the antiulcer activity of the extracts of *S.anacardium* using rats.

MATERIALS AND METHODS

Semecarpus anacardium seeds were purchased from Haryana Agricultural University, Hisar in Feb, 2009. The seeds were identified and authenticated by Dr. S. Sharma, Deptt. of Botany, HAU, Hisar..

Preparation of extracts

The air-dried *Semecarpus anacardium* seeds were powdered using a mechanical grinder. The dried powdered plant material (500 g) was refluxed with methanol (2L) and the semisolid dark brown mass was concentrated with rotary evaporator, yielding 80.50 g methanol extract (ME). The drug was treated with water (1.5 L), yielding 46.40 g aqueous fraction (AE).

Experimental Animals

The albino Wistar rats of either sex weighing 150-200 g were used for the study. Animals were divided into five groups, in each group six animals. They were maintained in the departmental animal house at 25 ± 2°C and relative humidity 45-55%, respectively for 1 week before and during the experiments. Animals were provided with standard rodent pellet diet (Hindustan Lever) and water *ad libitum*. The experimental protocol was approved by Institutional Animals Ethics Committee (IAEC) and animal care was taken as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India (Registration No. 0436).

Ethanol plus pylorus ligation method (EPPL)

The animals were placed in cages with grating floor to avoid coprophagy and divided into five groups each group six animal viz, Group I receiving 0.5% acacia gum served as vehicle control, group II ulcer induced group (EPPL), Groups III received aqueous extract at dose of 200 mg/kg and IV received the methanolic extract of *Semecarpus anacardium* seeds (MESA) at dose of 200 mg/ kg respectively by oral route. Group V received Cimetidine 40 mg/kg orally serving as standard drug control for EPPL model. All the extracts and reference drug were suspended in 0.5% acacia gum for animal administration. Ethanol was administered once daily for 5 days. Cimetidine and the extracts were administered 30 min before each ethanol administration. On day 6 after last dose, the rats were kept for 18 h fasting. Pylorus ligation was done by following the method as described. The animals were deprived of water during the postoperative period. After 4 h, stomachs were dissected out and contents were collected in tubes for estimation of biochemical parameters. Ulcers were scored as described.

Biochemical estimation

In EPPL induced ulcer models the following were estimated by procedures described, gastric volume, pH of gastric content, free acidity and total acidity. Dissolved muco substances such as total protein, hexoses, hexosamine, fucose, sialic acid were estimated in 90% alcoholic, precipitate of gastric juice and expressed as µg/ml.[21]

Statistical analysis

The data are expressed as mean ± SEM. Statistical comparisons were performed by one-way ANOVA followed by Dunnett's t test. The results were considered statistically significant if the *p*-values were less than 0.05.

RESULTS

Estimation of acid secretry parameters such as pH, gastric volume, free acidity and total acidity was increased significantly in the ethanol administered group. Administration of MESA a significant ($p < 0.01$) reduction in all the parameters and the results were comparable with the standard drug Cimetidine 40 mg/kg (Table 1). Determination of the concentrations of several muco proteins such as total protein, total hexoses, hexosamine, fucose and sialic acid revealed a decrease in ulcer induced group. The extract at 200 mg/kg increased the level of the muco proteins significantly and comparably with the standard drug ($p < 0.01$) (Table 2). The ulcer scores obtained in ulcer induced group of EPPL an increased score. Administration of the extract showed a significant decrease in EPPL models ($p < 0.05$ and $p < 0.01$). Table 3 shows Effect of MESA on level of SOD, LPO and CAT. The histopathological sections of the drug treated group EPPL had shown a reduction in ulcer focus and a hyperplastic gastric mucosa with regenerating mucosal epithelium (Figure 1, 2, 3 and 4).

DISCUSSION

MESA showed significant dose-dependent ulcer protective effect against ethanol plus pylorus ligation induced gastric ulcers. Ulcers are caused due to imbalances between offensive and defensive mucosal factors and hence the

Table 1: Effect of MESA on pH, gastric volume, free acidity and total acidity

Treatment group	pH	Gastric volume (mL/100g)	free acidity (m Eq/L/100g)	Total acidity (m q/L/100g)
Control	3.791 ± 0.1352	1.671 ± 0.1441	62.822 ± 0.2798	45.511 ± 0.4083
EPPL	1.223 ± 0.0736 a**	6.045 ± 0.1152 a***	72.304 ± 0.5827 a**	80.103 ± 0.2068 a
Aqueous extract *S. anacardium*	3.172 ± 0.1012 b**	4.733 ± 0.3575 b**	58.463 ± 0.7307 b**	57.322 ± 0.1536 b**
Methanolic extract *S. anacardium*	3.497 ± 0.8924 b**	3.542 ± 0.1612 b**	47.597 ± 0.4161 b**	53.014 ± 0.4081 b**
Cimetidine	3.862 ± 0.1352 b**	3.014 ± 0.1659 b**	44.061 ± 0.2382 b**	07.223 ± 0.2518 b**

Data are mean ± SEM., $n = 6$, Statistical significance *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, a - Group I vs. Group II; b indicates Group II vs. Groups III, IV and V

Table 2: Effect of MESA on gastric juice mucoprotein (µg/mL) in ELP rats

Treatment group	Protein	Total hexoses	Hexosamine	Fucose	Sialic acid
Control	264.92 ± 3.330	421.36 ± 5.678	207.27 ± 5.870	96.324 ± 1.656	72.983 ± 1.179
EPPL	473.36 ± 3.265 a***	195.51 ± 5.750 a***	110.21 ± 4.026 a***	6.347 ± 0.961 a***	21.161 ± 1.520 a***
Aqueous extract	399.38 ± 4.867 b**	399.38 ± 4.867 b**	162.19 ± 6.036 b**	81.423 ± 0.702 b***	48.322 ± 1.400 b**
MeOH extract	373.14 ± 11.91 b**	7.656 ± 6.085 b**	171.30 ± 4.973 b**	85.716 ± 1.073 b***	56.276 ± 0.869 b**
Cimetidine	329.83 ± 6.006 b**	403.14 ± 6.256 b**	175.53 ± 4.699 b**	116.14 ± 1.564 b***	64.096 ± 0775 b**

Data are mean ± SEM., $n = 6$, Statistical significance *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, a - Group I vs. Group II; b indicates Group II vs. Groups III, IV and V

Table 3: Effect of *S.anacardium* seeds extracts on induction of gross lesions in the ethanol plus pylorus ligation method (EPPL)

Group	Treatment	Dose orally (mg/ kg)	Ulcer index (Mean ± S.E.M)	Inhibition (%)
RR1	Control (0.5% Acacia gum)	200	0 49.157 ± 1.275	0
RR2	Absolute ethanol-HCl (ulcer control)	200	59.742 ± 1.655	0
RR3	Aqueous extract (AE)	200	49.157 ± 1.275*	30.42
RR4	Methanolic extract (ME)	200	28.959 ± 1.391**	71.06
RR5	Cimetidine	40	25.907 ± 1.485**	76.19

*$p < 0.05$ significant from (Absolute ethanol-HCl) ulcer control; **$p < 0.05$ significant from aqueous extract

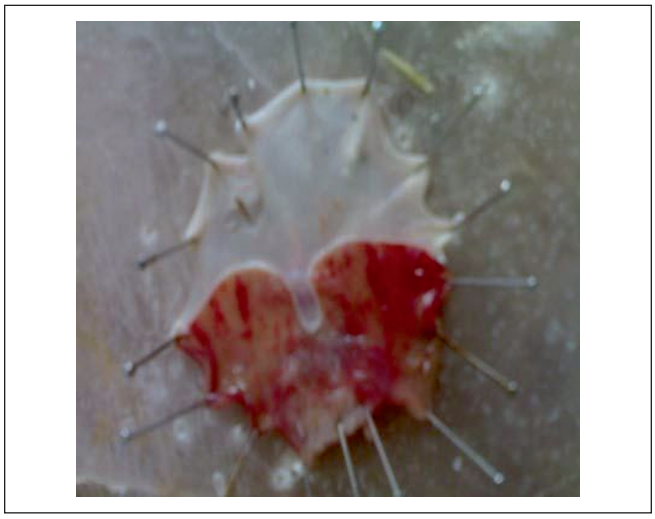

Figure 1: Control group

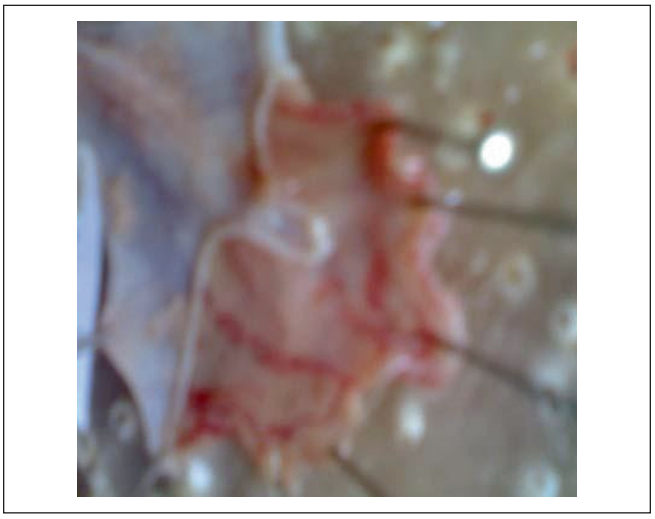

Figure 2: Aq. extract treated group

Figure 3: Methanol extract treated

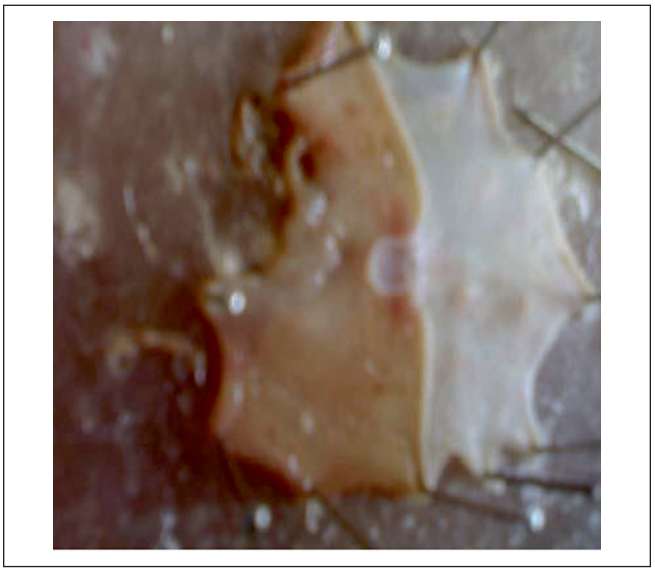

Figure 4: Cimetidine treated group

effects of MESA can be explained based on these factors. Mucin is a viscous glycoprotein with physiochemical properties producing relatively resistant acid barrier.[22] It makes up the major part of the mucus, an important pre-epithelial factor that acts as a first line of defence against ulcerogens. Increase in mucin can be due to increased levels of individual mucopolysaccharide like sialic acid and total hexoses. The increase in mucosal defence may also be due to decrease in cell exfoliation.[23] Hence, the protection afforded by MESA in EPPL induced ulcers may be predominantly due to strengthening mucosal defense. The ability of MESA to protect stomach against ulcerogens by neutralizing intra gastric acidity can as well lead it to classify as a cytoprotective agent. Prostaglandins have often been quoted as a model cytoprotective agent, although this has been disputed. To ascertain this effect, the activity of MESA was studied on Ethanol plus pylorus ligation model, where ethanol is known to further aggravate mucosal damage caused by pylorus ligation.[24] It was found that MESA was effective in this model, suggesting that the activity of MESA may also involve other defensive factors apart from PG synthesis. The role of the free radicals in gastric ulcerations is well-documented. MESA significantly reduced lipid peroxidation in rat gastric mucosa. *S. anacardium* has been reported to possess antioxidant activity. SOD scavenges the super oxide radical O_2–, one of the reactive oxygen species (ROS) responsible for lipid peroxidation. CAT and other peroxidases further reduce $H_2O_2^-$. The antioxidant activity of plant is already reported in literature. Thus the ulcer protective activity of MESA may also be due to its antioxidant effect. Hence, it can be suggested that MESA have antiulcer potential in rats and further in future, isolate the phytoconstituents which responsible for antiulcer activity may be studied.[21]

CONCLUSION

The present study reveals that the methanolic extract of *Semecarpus anacardium* seeds shows ulcer protective effect in ethanol plus pylorus ligation method. Hence, it can be suggested that methanolic extract(MESA) of *S. anacardium* have anti-ulcer potential in rats.

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Reducing of phorbol ester content in callus cultures of physic nut (*Jatropha Curcas* L.) using manganese chloride and n-ethylmaleimid

Komar Ruslan Wirasutisna[a], Artri[a], Elfahmi[a,b]

[a]*Pharmaceutical Biology Research group, Pharmaceitucal Science and Technology Study Program, School of Pharmacy, Bandung Institute of Technology, Jl. Ganesha 10 Bandung, Indonesia 40132*

ABSTRACT

Jatropha curcas L. (Euphorbiaceae) is an important plant. It has been used as pesticide, and herbal medicine. The oil from *J. curcas* can serve as biofuel that have high economically value. This plant contain toxic compound such as phorbol ester. Reducing of toxic compound from the plant would be useful to give added values of this plant. Callus cultures were initiated from *J. curcas* L. seed using MS medium, and treated by manganese chloride and n-ethylmaleimid. Phorbol ester content from seeds, callus and treated callus was measured by high performance liquid chromatography (HPLC). Content of phorbol ester in seed and callus culture were 7,41 mg/g DW and 2,44 mg/g DW - 5,27 mg/g DW, respectively. Treatment using manganese chloride ($MnCl_2$) 2 mM and 3 mM showed that the content of phorbol ester in callus culture decreased up to 30,5% and 30,6% respectively, at day 7. While, treatment using n-ethylmaleimid (NEM) 0,6; 0,9; and 1,2 mM showed that the content of phorbol ester in callus culture decrease up to 26,6%, 6,25%, and 32,2% respectively, at day 21. Phorbol ester content in callus culture of *J. curcas* was lower than its original seeds then it can be more reduced using manganese chloride and n-ethylmaleimid. This finding showed that cell cultures technique can be used to reduce the production of toxic compounds in *J. curcas*

Key words: cell culture, n-ethylmaleimid, *Jatropha curcas*, manganese chloride, phorbol ester, physic nut

INTRODUCTION

Jatropha curcas L. (physic nut) belonging to family Euphorbiaceae is a multipurpose tree. *J. curcas* can be used to make soap and pesticide. It has been used in traditional medicine for a long time, such as treatment for arthritis, gout, inflammation, dermatomucosal deseases, and toothache.[1] The oil from *J. curcas* can serve as fuel (biofuel) that have high economically value and alternative energy especially at this time. The levels of essential amino acids except lysine in *J. curcas* meal protein is higher than those of the FAO reference protein, so that *J. urcas* meal from nontoxic varieties could be a good source of protein for both livestock and human.[2] The oil also could be potential source for human consumption.[3] Because of its potential, *J. curcas* is already cultivated in various places and also potentially increase welfare of the society. Aspects that need to be considered in development of this plant is not only the potential use, but also aspects that ensure the safety of the consumption and processing. Because of seeds of *J. curcas* have toxicity such as irritant, causing vomiting, and diarrhea.

The toxicity of *J. curcas* seed links to chemical contained in. Beside curcin, phorbol esters is a family compounds known to cause a large number of biological effects such as produced a severely irritant reaction followed by necrosis (from testing on rabbits), can lead to death (from testing on mice),[4] and tumor promotion.[5] Toxicity of *J. curcas* seeds has been studied extensively in different animal models like sheep, goats, mice, rats, pigs, and fish when fed with phorbol ester-containing feeds.[6,7,8] For safety in use, phorbol esters that the main toxic agent in *J. curcas* seed should be decreased even eliminated. It is not possible to destroy phorbol esters by heat treatment because they are heat stable and can withstand roasting temperature as high as 160°C for 30 min. However, it is possible to reduce its

Address for correspondence:
School of Pharmacy, Institut Teknologi Bandung Indonesia (ITB), Jl Ganesha 10, Bandung 40116, Indonesia
Phone : +62-22-2504852
Fax : +62-22-2504852
E-mail: elfahmi@fa.itb.ac.id

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concentration in the meal by chemical treatments.[9] Besides detoxification by physically and chemically, detoxification may be developed with tissue culture method. This research was aimed to reduce the production of phorbol ester in *J. curcas* cultures. Tissue culture method would reduce the concentration of toxic compounds content by affecting the biosynthesis process of these toxic compounds. The use of substances as inhibitors in tissue culture methods was also expected to further inhibit the biosynthesis of phorbol esters.

MATERIALS AND METHODS

Plant material sterilization

Seeds of *J. curcas* were collected from Subang and Ciamis, West Java, Indonesia. Testa were removed from the seed. The seeds were washed with running tap water for 15 min. In LAF (Laminar Air Flow) cabinet, the seeds were then surface sterilized in commercial detergent (15% Bayclin) with 0.02% tween 20 for 15 min., followed by three rinses in sterile distilled water. Then seeds were cut into two part. Part of seeds were then surface sterilized in commercial detergent (10% Bayclin) with 0.02% tween 20 for 10 min., followed by three rinses in sterile distilled water.

Initiation of callus and maintenance

In the LAF cabinet, seed explants were cultured in MS medium supplemented with NAA (naphtalene acetic acid) 2.5 mg/ L and BAP (benzyl amino purine) 0.5 mg/ L. The media were congealed with agar (0.7%), and sucrose (2%) was used as a source of carbohydrate. All experiments were carried out in culture tubes containing 20 mL of culture medium. The pH of all media was adjusted to 5.7 ± 0.1 prior to autoclaving at 121°C for 20 min. Cultures were incubated at 25 ± 2°C. The callus was then maintained under the same condition as above with subcultures at 2 weeks interval in fresh medium. Callus that has been growing stable (approximately 5 times of subculture) were determined the rate of growth by weighing the mass of three callus from different culture tubes every 2 days for 30 days. Callus growth curve was stated by mass average of the three callus cultures and day of measurement.

Treatment of callus cultures

Optimal growth callus have been used for treatment using manganese chloride ($MnCl_2$) and n-ethylmaleimid (NEM). Treatments were started when the callus cultures had entered stationary phase. In treatment, callus was transferred to MS medium contained $MnCl_2$ or NEM in varying concentration. For each substance there were four treatment groups. Each group had three replicates. $MnCl_2$ treatment consisted of control, $MnCl_2$ 1, 2, and 3 mM group. N-etilmaleimid treatment consisted of control, NEM 0.6, 0.9, and 1.2 mM group. Each group was harvested at day 7th, 14th, and 21st. Callus that has been harvested and weighed were dried with a freeze dryer, measured the water content, weighed dry weight again, and analyzed the content of toxic substances. Effect of treatment was detected by analyzing the growth of callus and the content of toxic substances (phorbol ester).

Extraction of callus cultures

Amount of 0.2 g callus samples were weighed and powdered. Then callus were extracted with 10 mL of dichloromethane for 2 × 24 hours then filtered. Seven mL of filtrate were dried and then dissolved again with 0.5 mL of ethanol.

Quantification of phorbol ester with high performance liquid chromatography (HPLC)

Phorbol ester content was determined by modification of method developed by Makkar et al. (1997). The HPLC used Hewlett Packard 1100. The analytical column was reverse phase C18 (ODS Hypersil, 5 µm, 200 × 4.6 mm, Hewlett Packard). Two solvents were used: (A) 1.75 mL of o-phosphoric acid (85%) in 1 L of distilled water and (B) acetonitrile. The gradient elution was as follow: 60% A and 40% B at start, decrease A to 50% and increase B to 50% in the next 5 minutes, decrease A to 25% A and increase B to 75% in the next 10 minutes, increase B to 100% in the next 10 minutes, and then the column was adjusted to starting condition (60% A and 40% B) for 5 minutes. Separation was performed at room temperature and flow rate 1 mL/ min. The peak was integrated at 280 nm and the results were expressed as equivalent to phorbol-12-myristate 13- acetate (obtained from Sigma).

RESULTS

From the various medium compositions tested, MS medium containing 2.5 mg/L NAA and 0.5 mg / L BAP as growth hormones resulted in the best growth of callus of *Jatropha curcas*. This medium was used in all experiments. Initiation of callus generally occurs after 7 day. Callus was easily proliferated. Callus that formed was soft, compact, pale green (Figure 1). At the beginning of culture period, it was necessary to subculture after 2 weeks, otherwise the callus became dark and reduced growth. Based on growth curve during 30 days, callus culture was rapidly growing until day 18, From day 18-22, callus was growing slowly, then reach optimum growth at day 22. There were two type of callus with different color (brown and green callus). Callus cultures treated with 0.6 mM NEM was still growth although the rate was lower than control. Callus culture treated with NEM of 0.9 and 1.2 mM showed a decrease of growth after 14 day (Figure 2). While the growth of callus culture treated with various concentration of $MnCl_2$ showed better

growing compared to control. Slope of the treated callus culture is greater than the control. It can be indicated that $MnCl_2$ addition in the media increased the growth of callus cultures, at a concentration of 1 mM, 2 mM or 3 mM (Figure 3).

We found qualitatively and quantitatively different chemical content profiles comparing seed from different collection, callus culture control, callus treated with NEM and callus treated with $MnCl_2$. Analysis with HPLC showed that phorbol esters were detected in the retention time range 21.2-21.8 min. Phorbol ester content in callus culture (2.4-5.3 mg/g DW) was lower than the original seed (7.4-7.7 mg/g DW) (Figure 4). These result showed that tissue culture methods could significantly decrease phorbol ester content. Differences content of phorbol ester between green and brown callus were probably due to the presence of other compounds that affect to the production of phorbol ester. From the curve were observed, the content of phorbol ester in seed origin from Subang (used as explants) and Ciamis showed little difference in levels.

Phorbol ester content in control callus culture decreased slightly at 7th day, while at 14th day phorbol ester content decreased sharply (Table. 1). Phorbol ester content from callus treated with 1 mM $MnCl_2$ did not differ significantly to callus control at day 7, 14 and 21. Callus culture treated with 2 mM of $MnCl_2$ reduced the levels of phorbol ester 30 and 10%, at day 7 and 14 compared to the control (4.83 and 2.26 mg/g DW) respectively, while at day 14 no significant reduction reached. Callus culture treated with 3 mM of $MnCl_2$ reduced the level of phorbol ester only at day 7 (30%), while at day 14 and 21 did not reduce phorbol ester content. Callus culture treated with 3 mM $MnCl_2$ were still growing well, indicated that $MnCl_2$ was not toxic to the callus culture up to the concentration used. Phorbol

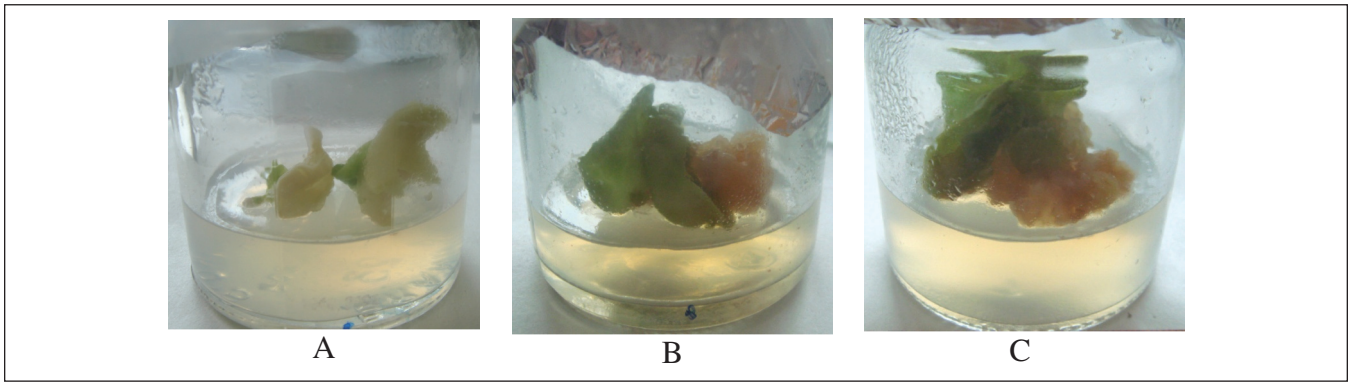

Figure 1: Callus formation of *Jatropha curcas* from seed explants after: A) 8 days; B) 25 days and C) 36 days of culture on MS medium supplemented with 2.5 mg/ L NAA and 0.5 mg/ L BAP

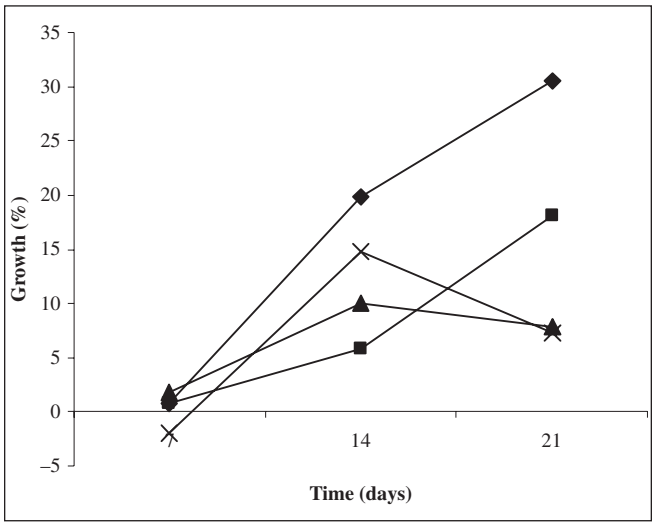

Figure 2: Growth curve of callus culture treatment with NEM ◆ = kontrol, ■ = 0,6 mM NEM, ▲ = 0,9 mM NEM, X = 1,2 mM NEM

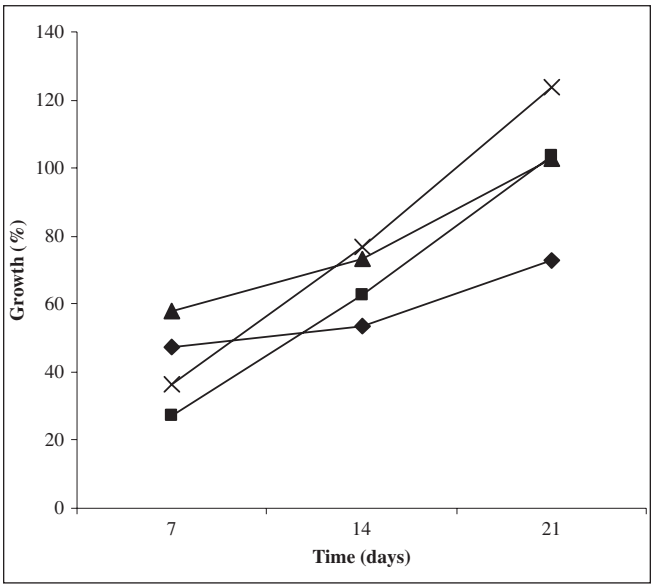

Figure 3: Growth curve of callus culture on treatment with $MnCl_2$ ◆ = control, ■ = 1 mM $MnCl_2$, ▲ = 2 mM $MnCl_2$, X = 3 mM $MnCl_2$

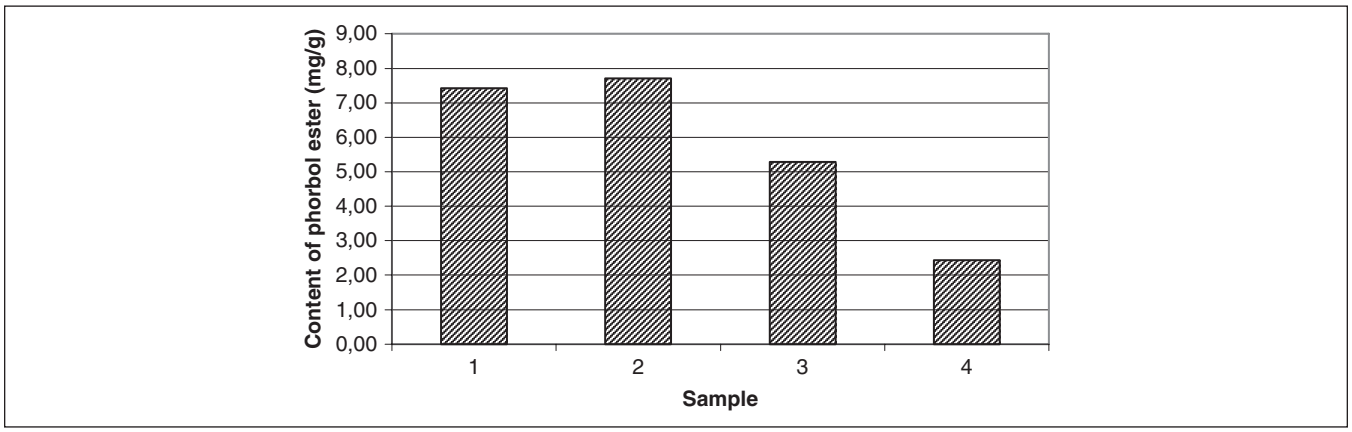

Figure 4: Phorbol ester content of 1) seed sample collected from Subang, 2) seed sample collected from Ciamis, 3) Green callus culture and 4) Brownish callus culture of *Jatropha curcas*

Table 1: Phorbol ester content on callus culture treated with $MnCl_2$

Day	Sample of callus culture of control and treated with $MnCl_2$ (mM)	Phorbol ester content (mg/g DW)
0	Control	5.27 ± 0,41
7	Control	4.83 ± 0.09
	1	4.58 ± 2.72
	2	3.36 ± 0.20
	3	3.35 ± 0.87
14	Control	2.26 ± 0.15
	1	2.04 ± 0.43
	2	2.08 ± 0.03
	3	2.31 ± 0.11
21	Control	2.12 ± 0.30
	1	2.11 ± 0.22
	2	2.09 ± 0.22
	3	2.28 ± 0.25

Table 2: Phorbol ester content on callus culture treated with n-ethylmaleimid (NEM)

Day	Sample of callus culture control and treated with NEM (mM)	Phorbol ester content (mg/g DW)
0	Control	2.44 ± 0,26
7	Control	2.19 ± 0.59
	0.6	2.37 ± 0.03
	0.9	3.22 ± 0.77
	1.2	3.20 ± 0.61
14	Control	1.54 ± 0.08
	0.6	1.10 ± 0.12
	0.9	1.48 ± 0.06
	1.2	2.03 ± 0.49
21	Control	21.85 ± 2.62
	0.6	16.04 ± 2.86
	0.9	20.48 ± 6.02
	1.2	14.80 ± 7.21

ester content in control and treatment treated with NEM callus culture was linear from day 0 to day 14, further increased sharply after the 14th day. Observing the curve from day 14 until day 21 showed that the addition of NEM can reduce levels of phorbol ester. Content of phorbol ester in callus culture treated with 1.2 mM NEM was reduced up to 32%.

DISCUSSION

Tissue culture is one tool that can be used to modify the content of compounds in the original plant. In this experiment, seed was selected for explant. The best seeds for explants are quite young, which seed coat characteristics (testa) is black and still covered white pericarp. Seeds that are too young or too old are generally not will grow. Based on mass measurements of callus during 30 days (Figure 1), it is known that callus cultures grew rapidly from day 0 to day 18, then the growth slowed until day 22 and peak growth occurred at 22th day. After day 22, the growth of callus culture had been decreased. The callus culture would subcultured after 30 day, otherwise the callus culture became dark.

Feeding experiment with NEM slightly inhibited growth of callus callus culture at the higher concentration, therefore we assume that administration of 0.9 and 1.2 mM NEM is toxic to callus culture. The callus culture also became dark. While feeding experiment with $MnCl_2$ enhanced the growth of callus culture. Pale green callus was used for treatment in media containing excess $MnCl_2$. Actually $MnCl_2$ was contained on MS media, since this substance is a micronutrient needed for plant growth. Treatment of callus cultures with $MnCl_2$ reduced phorbol ester content up to 32%. We assume that $MnCl_2$ inhibited the biosynthesis of phorbol ester which is synthesized via IPP and DMAPP pathway. This is supported by several previous report from various publications. Mn^{2+} is a cofactor that catalyzes isomerization reaction between IPP and DMAPP.[10] In vitro studies that tested the Mn^{2+} with IPPs that were extracted from the seeds of *Ricinus communis*, tomatoes

plastid, pork liver, and microbes, Mn^{2+} active as a cofactor at a concentration about 0.5 mM. Meanwhile, at a concentration of more than 1 mM Mn^{2+} are strongly inhibitors.[11] Another study mentioned that a high concentration of Mn^{2+} disrupt the bond between the substrate with the enzyme.[12] The treatment was also carried out with n-etilmaeimid (NEM) on brownish callus. NEM is a compound that is able to bind to enzymes which have sulfhydryl groups such as cystein amino acid. That binding can inactivate enzymes. Based on the literature, NEM can inhibite IPP isomerase enzyme, geranil transferase, and farnesil transferase activity.[10,11] Since both compounds were found to have influence in the biosynthesis secondary metabolites through IPP and DMAPP pathway, they were chosen as inhibitor of phorbol esters. These toxic compounds belonging to diterpen were synthesized via IPP and DMAPP pathway. Therefore, feeding both compounds to callus culture was expected to reduce the production of phorbol esters.

Different content of phorbol ester in *J. curcas* seed collected from differences place (Subang and Ciamis, West Java, Indinesia) suggested that the phorbol ester content could be influenced by ecological factors such as temperatures, moisture and seasons. Different content of phorbol ester were also found between seed and callus culture. This may be caused the different profile of biosynthesis condition between seed and callus culture. These results supported our previous research on the production of secondary metabolites which were different to the original plants.[13] Differences pattern phorbol ester content in control treated with NEM and $MnCl_2$ can be caused due to different callus culture as a source, callus culture on $MnCl_2$ treatment used pale green callus culture, while in the NEM treatment used brownish callus culture.

Inhibition of phorbol ester production by $MnCl_2$ was found at day 7 with concentration 2 and 3 mM. The inhibition was not time-dependent which can be clearly explained its reason. It may be due to the activity of enzyme involved in the phorbol ester biosyhntesis. This fact was also shown by inhibition of phorbol ester production by NEM which was optimal at day 21 with concentration of NEM 1.2 mM. In future this result can be used as a basis to generate the new strain of *J. curcas* which contain low level of toxic compounds.

CONCLUSION

Phorbol ester content in callus culture of *J. curcas* was lower than its original seeds than it can be more reduced using manganese chloride and n-ethylmaleimid as inhibitors of biosynthesis of diterpen compounds which phorbol ester belonging to. This finding showed that cell cultures technique can be used to reduce the production of toxic compounds in *J. curcas*

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DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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In-vitro studies on the antioxidant assay profiling of *Withania somnifera* L. (Ashwagandha) Dunal root: Part 1

Ajay Pal*, Mahadeva Naika, Farhath Khanum and Amarinder Singh Bawa

Biochemistry and Nutrition Discipline, Defence Food Research Laboratory, Siddarthanagar, Mysore – 570 011, India

ABSTRACT

Withania somnifera Dunal, commonly known as Ashwagandha, has been used for centuries in Ayurvedic medicine to increase longevity and vitality. Various groups of researchers support its polypharmaceutical uses by confirming anti-inflammatory, immuno-modulatory and antistress properties in the whole plant extract. The present investigation adds up the antioxidant assay profiling in this list. The Ashwagandha root extracts were prepared in different solvents of varied polarity in a sequential fashion. The maximum yield (5.8%) and total polyphenolic compounds (TPC, 88.58 µg GAE/mg extract) were recorded in methanolic fraction. All the extracts were screened for their potential antioxidant activities using tests such as free radical scavenging activity, metal chelation activity, hydroxyl radical scavenging activity, superoxide radical scavenging activity and hydrogen peroxide scavenging activity. Results of study showed methanolic extract as the most potent fraction and a strong correlation could be established with its TPC. Overall, the study suggests that Ashwagandha root extract could be of great use for the preparation of antioxidant rich nutraceuticles to treat various human diseases and its complications. It is quite likely that the antioxidant capacity of plant/root is one of the reasons for its established anti-stress, anti-inflammatory and immunomodulatory properties.

INTRODUCTION

Recent studies have shown that oxidative stress plays a very important role in the etiology of several diseases and antioxidants might be useful in their treatment.[1-3] Increase in oxidative stress as a result of an imbalance between free radical production and antioxidant defense has been well documented in several clinical studies.[4] In humans, strenuous exercise promotes the generation of reactive oxygen species (ROS) in the working muscle. The growing number of evidences indicates that these ROS are responsible for exercise-induced protein oxidation and contribute significantly to muscle and mental fatigue. It has been postulated that treatments that reverse these ROS generated injuries may be acting through mechanisms that scavenge these species.[5]

Flavonoids, tannins and other phenolic constituents of plant food have been proved as potential antioxidant moieties.[6] During certain diseased conditions, as well as during aging, there is a need to boost the antioxidant capacity of body to potentiate the immune mechanism. The antioxidants preserve the adequate function of neuronal cells against homeostatic disturbances.[7]

Herbal medicine (herbalism or botanical medicine) or the use of herbs for their therapeutic or medicinal value is the oldest form of healthcare.[8-9] Recent years have seen a surge of interest in the use of herbal drugs to treat various ROS mediated complications because of their safety, efficiency and cost effectiveness.[10] But, the evaluation of these properties of herbs or herbal preparations requires an understanding of its potential to scavenge ROS and enhance antioxidant defense in the body.

The Indian medicinal plant *Withania somnifera* (L) Dunal (family- solanaceae), commonly known as Ashwagandha, is widely used in herbal medicine for stress, arthritis, inflammations, conjunctivitis and tuberculosis.[11-15] The active principles of Ashwagandha, consisting of sitoindisides VII-X and withaferin-A have been shown to exhibit significant antistress and antioxidant effect in rat brain frontal cortex and striatum.[16] Although, many of these medicinal properties of Ashwagandha are attributed to its

Address for correspondence:
Tel.: + 91 821 2474676; fax: + 91 821 2473468.
E-mail: ajaydrdo@rediffmail.com (A. Pal)

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antioxidant compounds yet there is no detailed report available on its antioxidant profiling assays. Our laboratory is actively engaged in the development of performance enhancing foods to be used by our armed forces. In the preliminary phase of study, the present manuscript delineates the antioxidant properties of Ashwagandha root extract based on its ability to scavenge various free radicals.

MATERIALS AND METHODS

Preparation of sample extract

The Ashwagandha roots were obtained from the garden of Government Ayurvedic Medical College, Mysore, India. The antioxidant compounds from dried and powdered root were extracted by adding solvents in increasing order of their polarity, namely hexane (H), chloroform (C), ethyl acetate (EA), acetone (A), methanol (M) and water (W) in a sequential manner. After filtering through folded Whatman No. 1 filter paper, the supernatant in different solvents was recovered and this process was repeated thrice with each solvent. Then, the respective solvents from the supernatant were evaporated in a vacuum rotary evaporator to obtain the yield of different extract. For checking the antioxidant activity, each extract/fraction was dried and redissolved in dimethylsulfoxide (DMSO).

Determination of total phenolic content (TPC), flavonoids, flavonols and tannins

The total phenolic content (TPC) of different extracts was determined by the method of Folin-Ciocalteu using gallic acid as the standard.[17] A calibration curve was made for gallic acid and the results were determined from regression equation of this calibration curve, which was expressed as gallic acid equivalent (GAE) in μg/mg extract. To 3 ml of appropriately diluted extract was added 0.5 ml of (50%) Folin-Ciocalteu reagent, followed by incubation at room temperature (10 min) and addition of 7% Na_2CO_3 (2 ml) solution. The mixture was boiled for 1 min, cooled and the absorbance was measured at 650 nm.

The determination of flavonoids was carried out according to Delcour and Varebeke.[18] To 1 ml of appropriately diluted extracts, 5 ml of chromogen reagent (0.1% cinnamaldehyde solution prepared in a cooled mixture of 25 ml of concentrated HCl and 75 ml of methanol) was added. After an incubation of 10 min, the absorbance was measured at 640 nm. The total flavonoid content was expressed as catechin equivalents (CE) in μg/mg extract.

The content of flavonols was determined by the method of Yermakov et al.[19] The rutin calibration curve was prepared by mixing 1 ml of various concentrations of rutin solutions with 1 ml (40 mg/ml) aluminium trichloride and 3 ml (100 mg/ml) sodium acetate. The absorbance at 440 nm was read after 2.5 h. The same procedure was used for plant extract and the total flavonols content was expressed as rutin equivalents (RE) in microgram per milligram extract.

For the estimation of tannin, 2 ml of different concentrations of extract was treated with 5 ml of Folin-Denis reagent and 10 ml of saturated solution of Na_2CO_3. The mixture was allowed to incubate at room temperature for 30 min and absorbance was measured at 700 nm.[20]

DPPH radical scavenging assay

The extracts were measured in terms of their hydrogen-donating or radical-scavenging ability using the stable radical, DPPH*, following the method of Blois[21] with slight modifications. Briefly, the reaction mixture contained 3 ml of appropriately diluted extract in methanol and 0.5 ml of DPPH* (500 μM methanolic solution). The reaction mixture was allowed to stand in dark at room temperature for 45 min and absorbance was recorded at 515 nm against the methanol blank. A control was taken without plant extracts under identical conditions. The percent free radical scavenging capacity (%RSC) of the extracts was calculated from control and IC_{50} from linear regression analysis. BHA was used as a standard antioxidant.

Metal chelation assay

The chelating effect of extracts on ferrous ions was determined according to the method of Dinis et al.[22] with some modifications. The appropriately diluted extract (2 ml) was mixed with 0.05 ml of 2 mM $FeCl_2$. This was followed by the addition of 0.2 ml of 2 mM ferrozine, which was left to react at room temperature for 10 min before determining the absorbance of the mixture at 562 nm. The inhibition percentage of ferrozine–Fe^{2+} complex formation was calculated from control without sample under similar conditions. The results were expressed as IC_{50} calculated from linear regression analysis. EDTA was used as a standard antioxidant.

Hydroxyl radical scavenging assay

The assay was performed following the method of Halliwell et al.[23] with slight modifications. The reaction mixture contained 2-deoxyribose (0.1 ml of 28 mM), 1 ml solution of various concentrations of extracts, 0.2 ml of mixture solution containing equal amount of 200 μM $FeCl_3$ and 1.04 mM EDTA, 0.1 ml of H_2O_2 (1.0 mM) and 0.1 ml ascorbic acid (1.0 mM). The mixture was then incubated at 37°C for 1 h and boiled for 20 min with 1 ml of 1% TBA in 50 mM NaOH and 1 ml of 5% trichloroacetic acid (TCA) in a water bath. After cooling, absorbance of the mixture was measured at 532 nm. The % inhibition was calculated from control without sample under similar

conditions. IC_{50} was calculated from linear regression analysis Gallic acid was used as a standard antioxidant.

Superoxide anion radical scavenging assay

The measurement of superoxide anion scavenging activity of different extracts was based on the modified method of Liu et al.[24] The method is based on the generation of superoxide radicals in PMS–NADH system by oxidation of NADH and subsequent assay of reduced NBT. In this experiment, the superoxide radicals were generated in 3 ml of Tris–HCl buffer (16 mM, pH 8.0) containing 1ml of NBT (150 µM) solution, 1ml NADH (468 µM) solution and sample/extract. The reaction was started by adding 1ml of PMS solution (60 µM) to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance at 560 nm was measured. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The inhibition percentage of superoxide anion generation was calculated using control without sample under similar conditions. IC_{50} were calculated using regression analysis. L-ascorbic acid was used as the standard.

Hydrogen peroxide scavenging assay

The ability of different extracts to scavenge hydrogen peroxide was determined according to the modified method of Ruch et al.[25] A solution of hydrogen peroxide (10 mM) was prepared in 100 mM phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm with molar absorbtivity 81 $(mol/l)^{-1}$ cm^{-1}. Sample/standard in distilled water was added to hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution. The percent inhibition activity was calculated from control without extract under similar conditions. IC_{50} was calculated from regression analysis. L-ascorbic acid was used as a standard antioxidant.

RESULTS AND DISCUSSION

Ashwagandha is one of the most commonly used herbs as an anti-stress and adaptogenic agent. Besides anti-inflammatory, anti-tumour, anti-stress, immunomodulatory and hematopoietic properties, it is also known to increase life span and delay ageing. The Ashwagandha roots contain several alkaloids, withanolides, a few flavonoids and reducing sugars and are also rich in iron.[26] The major active compounds of the roots are reported to be withanolides, glycosides and many different alkaloids.

Since oxidation-antioxidation process is very complex, it is quite clear that no single testing method can provide a comprehensive picture of the antioxidant profile of a given herb. Therefore a multi-method approach has been recommended to assess the antioxidant potential of a sample. In present investigation, the extracts of the Ashwagandha root were prepared in different solvents of varied polarity and antioxidant activities of all the extracts were evaluated in different assay systems *viz.* DPPH* assay, metal chelation assay, superoxide scavenging assay, hydroxyl radical scavenging assay and hydrogen peroxide scavenging assay. Taking 0% inhibition in the assay mixture devoid of extract, linear regression equations were generated from a plot between the concentrations of the extracts and percentage inhibition of free radical formation/prevention. The obtained equations were used for the calculation of IC_{50} values (concentration of sample required to scavenge 50% free radical). The IC_{50} values are inversely related to the activity.

Extraction efficiency

Six solvents from non-polar (hexane) to polar (water) were used sequentially to extract compounds from Ashwagandha roots and the corresponding yield is presented in Figure-1. The extraction experiments revealed that 100% methanol was the best solvent for the extraction of compounds as it recorded a maximum yield of 5.8%. A minimum yield of 0.45% was recorded in ethyl acetate fraction. Because methanol is a relatively polar organic solvent compared to other extracting solvents like hexane, chloroform, ethyl acetate and acetone, it can be concluded that compounds present in Ashwagandha root are most likely polar in nature.

Various reports on the extraction of compounds in different solvents have shown methanol as better extractant since methanol is more efficient in cell wall degradation as compared with other solvents.[27] A minimum of 70% methanol has been found needed to inactivate polyphenol oxidases (PPO), which might produce qualitative and

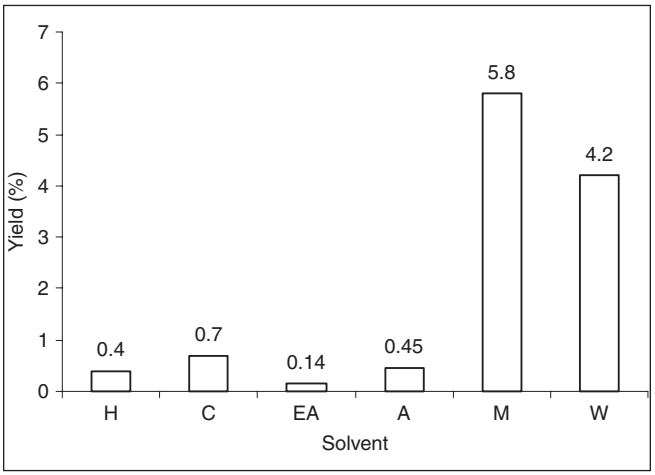

Figure 1: Yields of Ashwagandha root compounds in different solvents

quantitative changes in the phenolic content of extract. The phenolics, once migrated in the extract, act synergistically to alter the fluidity of outer and inner membrane and results in a release of cell materials thereby influencing the yield.[28-30]

TPC, flavonoids, flavonols and tannins contents

Literature pertaining to plant phenolic studies indicates that they constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators. Therefore, it is worthwhile to determine their content in the plant extracts under study. Flavonoids, being one of the most diverse and widespread group of natural compounds, are likely to be the most important natural phenolics.[31] These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties. Such properties are especially distinct for flavonols and tannins. Therefore, the content of all the groups of phenolics was also determined in the extracts. Another objective behind their estimation was to establish relationships with different antioxidant activities.

The TPC values of the different extracts determined using the Folin–Ciocalteu assay, ranged from 4.96 to 88.58 µg GAE/mg of extract (Table-1). Differences in the polarity of the extracting solvents could result in a wide variation in the polyphenolic contents of the extract. Considering all the fractions, the methanol extract was found to contain the highest phenolic content. The lowest amount of phenolics was found in the hexane fraction. Based on the findings it can be inferred that most polyphenolics evaluated in this study are likely polar compounds.

Table 1: TPC, flavonoids, flavonols and tannins in different extracts of Ashwagandha root

Extract	TPC	Flavonoids	Flavonols	Tannins
	(µg/mg extract)			
Hexane	4.96	0.19	0.11	1.86
Chloroform	42.00	0.52	0.29	19.76
Ethyl acetate	61.09	0.82	0.45	29.86
Acetone	45.31	0.49	0.28	27.24
Methanol	88.58	0.85	0.48	37.03
Water	66.72	0.38	0.21	16.96

Since flavonoids are a class of secondary plant phenolics with powerful antioxidant properties,[32] it would be valuable to determine the flavonoid contents of the different extracts under study. The phytochemical investigations of different extracts of Ashwagandha demonstrated the presence of maximum and minimum flavonoids in methanolic (0.85 µg CE/mg of extract) and hexane (0.19 µg CE/mg of extract) extracts, respectively. Also, the amount of flavonols and tannins corresponded well with TPC and flavonoids being maximum and minimum in methanol and hexane fractions, respectively.

Radical scavenging capacity (RSC)

The bleaching of DPPH absorption (517 nm) by a test compound is representative of its capacity to scavenge free radicals, generated independent of any enzymatic or transition metal-based systems. Hence, the DPPH-scavenging activities of the extracts were taken as the parameter to check their antioxidant potential. A dose dependent increase in quenching of free radical was observed for all the extracts. The linear regression equations used for calculation of IC_{50} values of different extracts are shown in Table-3. The RSC of the different extracts is shown in Figure-2. The best free radical scavenging activity was shown by the methanolic fraction (IC_{50} = 55.71 µg/ml), followed by water (IC_{50} = 94.12 µg/ml) and ethyl acetate fractions (115.16 µg/ml). The hexane fraction was found to be least potent as indicated by its highest IC_{50} value (404.28 µg/ml). Among all extracts, the highest antioxidant content (TPC, flavonoids, flavonols, tannins) and RSC was found for the methanol extract and some good correlations were found among these parameters (Table-2).

It is important to examine these correlations between antioxidant content and RSC of the extracts because some authors have reported that there is no correlation between the content of these main antioxidant compounds and the RSC.[33] The results obtained by us do not support these claims. Table-2 shows that the highest correlation coefficient was exhibited between the TPC and the RSC (correlation coefficient, R^2, 0.932) and the lowest correlation coefficient of RSC was found with flavonols and flavonoids content. These data are in accordance with others, who have shown that high TPC increases the antioxidant activity[34-35] and

Table 2: Correlation coefficient (R^2) between antioxidant contents and antioxidant activities

Antioxidant activity	Antioxidant contents			
	TPC	Flavonoids	Flavonols	Tannins
RSC	0.932	0.543	0.539	0.669
Metal chelation activity	0.788	0.517	0.513	0.712
HRS activity	0.765	0.480	0.477	0.706
SRS activity	0.760	0.505	0.501	0.705
Hydrogen peroxide scavenging activity	0.886	0.345	0.348	0.568

there is a linear correlation between phenolic content and antioxidant activity.[36] The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides.[37] It can be observed that the content of phenolics in the extracts correlates with the antioxidant activity, but such a correlation with flavonoid or flavonols content is not obtained. The findings indicate that more than 90% RSC activities of different extract can be attributed primarily to their TPC content.

Metal chelating activity

Iron is essential for life because it is required for oxygen transport, respiration and the activity of many enzymes. However, it is an extremely reactive metal and causes the oxidative damage to lipids, proteins and other cellular components.[38] Therefore, ability of the different extracts to chelate/bind metal ion was tested. The method for this activity is based on chelating of Fe^{+2} by the reagent ferrozine, which is a quantitative formation of a complex with Fe^{+2}.[22]

Table 3: Regression equations and correlation coefficients for FRS activity of different extracts of Ashwagandha

Extract	Regression equation	Correlation coefficient (R^2)
Hexane	y = 0.1143x + 3.79	0.9847
Chloroform	y = 0.201x – 6.865	0.9861
Ethyl acetate	y = 0.4138x + 2.3429	0.9894
Acetone	y = 0.2296x + 5.5518	0.986
Methanol	y = 0.782x + 6.429	0.9732
Water	y = 0.4523x + 7.4291	0.9779

The formation of complex is probably disturbed by the other chelating agents, which result in the reduction of formation of red-coloured complex. Measurement of the rate of reduction of the color, therefore, allows the estimation of chelating activity of the co-existing chelators. In the assay, all the extracts intervened with the formation of ferrous complex with the reagent ferrozine, suggesting that it has chelating effects and captures the ferrous ions before ferrozine. The absorbance of Fe^{+2}-ferrozine complex linearly decreased with the increase in concentration of extracts. The regression equations were generated for the calculation of IC_{50} values which are summarized in Table-4. Herein Figure-3 shows the chelation activity of different extracts which shows that the IC_{50} values of the extracts follows the order: methanol (69.89 μg/ml) > water (93.49 μg/ml) > ethyl acetate (113.53 μg/ml) > acetone (145.46 μg/ml) > chloroform (162.83 μg/ml) > hexane (626.06 μg/ml). The data clearly demonstrate that the methanolic extract of root possesses effective capacity for iron binding. The R^2 between the metal chelation activity and the TPC/flavonoids/flavonols/tannins content of the different extracts was determined (Table-2). The chelation activity showed a better correlation with the TPC ($R^2 = 0.788$) and tannins ($R^2 = 0.712$) than flavonoids ($R^2 = 0.517$) and flavonols contents ($R^2 = 0.513$). Therefore, it can be inferred that phenolic compounds are the dominant contributor to the metal chelation activity of Ashwagandha root. The metal chelating capacity is of prime importance as it reduces the concentration of transition metal involved in lipid peroxidation. It has also been proved that chelating agents, which form sigma bonds with a metal, are effective as secondary antioxidants since they reduce the redox potential thereby stabilizing the oxidized form of the metal ions.[39]

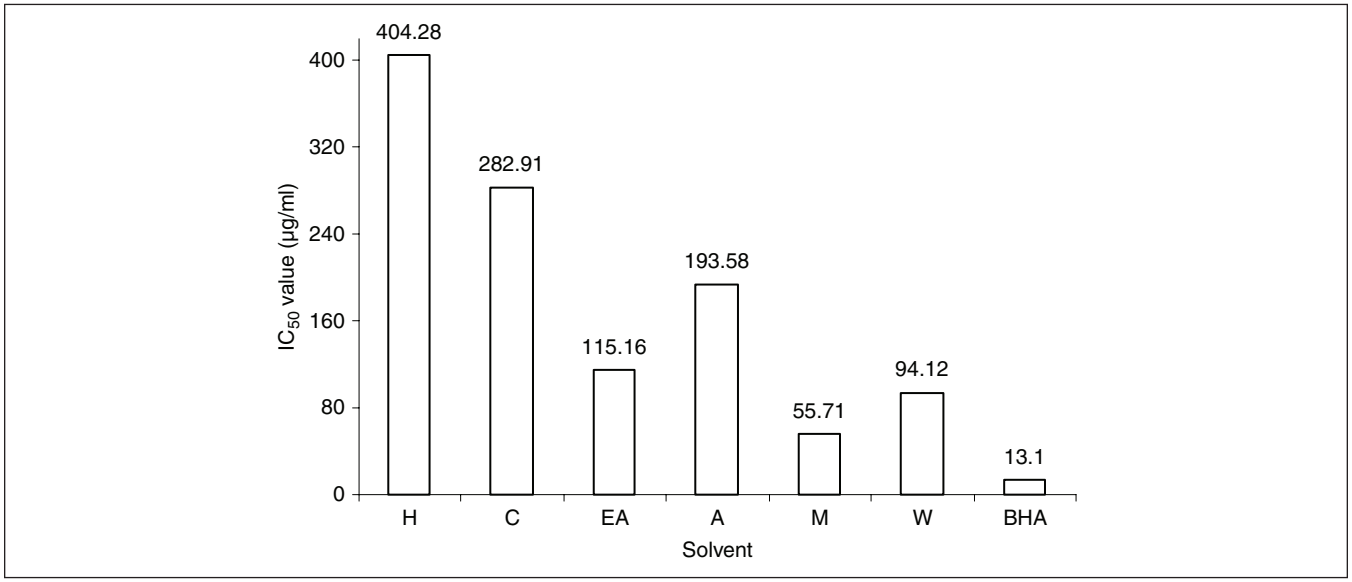

Figure 2: Comparison of DPPH radical scavenging activity of different extracts of Ashwagandha root

Hydroxyl radical scavenging (HRS) activity

Among the ROS, the hydroxyl radicals are the most reactive and predominant radicals generated endogenously during aerobic metabolism.[40] Hydroxyl radical is an extremely reactive oxygen species, capable of modifying almost every molecule in the living cells. This radical has the capacity to cause strand damages in DNA leading to carcinogenesis, mutagenesis, and cytotoxicity. Moreover, hydroxyl radicals are capable of the quick initiation of lipid peroxidation process by abstracting hydrogen atoms from unsaturated fatty acids.[41-42]

Due to their high reactivity, the radicals have a very short biological half-life. Thus, its effective scavenger must be present at a very high concentration or possess very high reactivity towards these radicals. Consequently, the ability of all extracts to scavenge these radicals was evaluated by the 2-deoxyribose assay. When hydroxyl radical, generated by the Fenton reaction, attacks deoxyribose it degrades into fragments that react with TBA on heating at low pH to form a pink color. All the extracts showed a dose dependent response towards chelation of hydroxyl free radical. The resulting linear regression equations are shown in Table-5. The best results, expressed in terms of IC_{50} value, were exhibited by the methanol extract with minimum IC_{50} value of 0.57 mg/ml (Figure-4). Relatively good results were also exhibited by the water extract (IC_{50} = 0.80 mg/ml) and the lowest activity was found in the hexane fraction. These results clearly demonstrated the capacity of methanolic extract to quench hydroxyl radicals.

Table 4: Regression equations and correlation coefficients for metal chelating activity of different extracts of Ashwagandha

Extract	Regression equation	Correlation coefficient (R^2)
Hexane	y = 0.0787x + 0.7289	0.9889
Chloroform	y = 0.2918x + 2.4848	0.9693
Ethyl acetate	y = 0.4556x – 1.725	0.989
Acetone	y = 0.3176x + 3.8	0.9642
Methanol	y = 0.7205x – 0.3619	0.9974
Water	y = 0.5137x + 1.9714	0.9856

A fair degree of correlation (R^2 = 0.76) was found between TPC and HRS activity while the lowest correlation coefficient was found with flavonols (Table-2). The results suggest that polyphenolic compounds present in different extracts are probably involved in their HRS activity.

Superoxide radicals scavenging (SRS) activity

The superoxide radicals, despite of being involved in many pathological processes, by themselves are not as reactive as the hydroxyl radicals. But they can give rise to toxic hydroxyl radicals, damaging cellular macromolecules directly or indirectly with severe consequences.[43] The superoxide radicals have been proved to play crucial roles in ischemia-reperfusion injury.[44] Thus, scavenging of these radicals would be a promising remedy for this disease.

The scavenging activity of different extracts for superoxide radicals generated by a PMS/NADH system was assessed by measuring the absorbance at 560 nm.[45] The IC_{50} values were calculated using the regression equations listed in Table-6. Figure-5 shows the IC_{50} values of various extracts against superoxide radical anions. The highest SRC activity was exhibited by methanolic extract followed by water extract

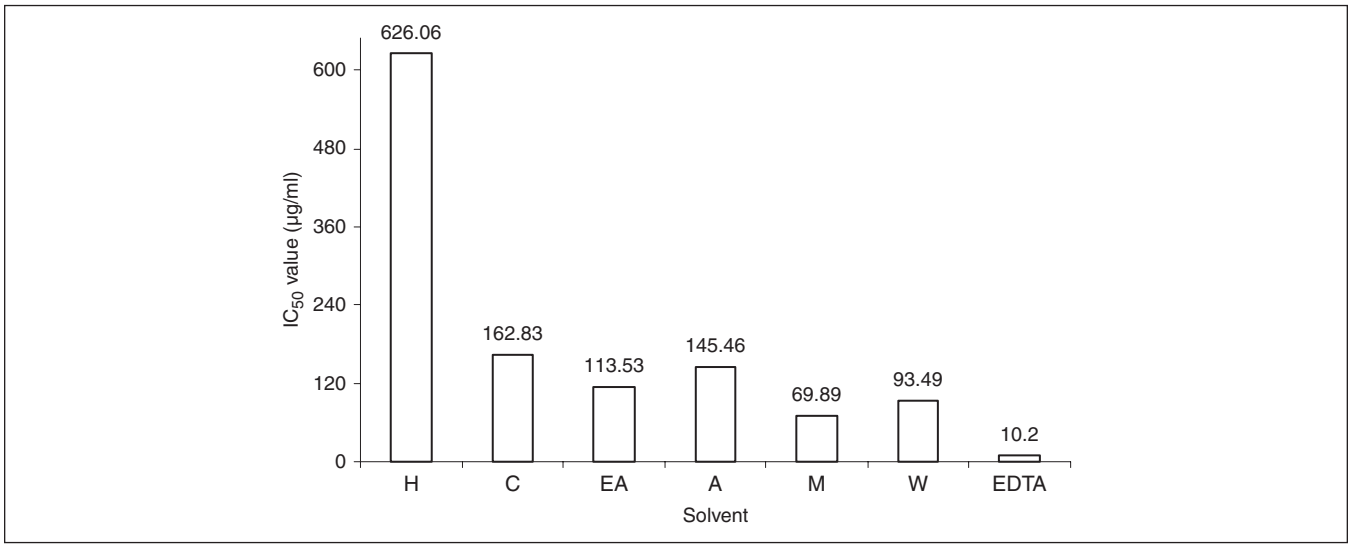

Figure 3: Comparison of metal chelation activity of different extracts of Ashwagandha root

Table 5: Regression equations and correlation coefficients for HRS activity of different extracts of Ashwagandha

Extract	Regression equation	Correlation coefficient (R^2)
Hexane	y = 5.247x + 1.0333	0.9839
Chloroform	y = 23.89x – 0.6583	0.9895
Ethyl acetate	y = 0.0372x – 0.8143	0.9735
Acetone	y = 0.0419x – 1.8929	0.9853
Methanol	y = 0.087x + 0.5571	0.9948
Water	y = 0.0651x – 1.925	0.9863

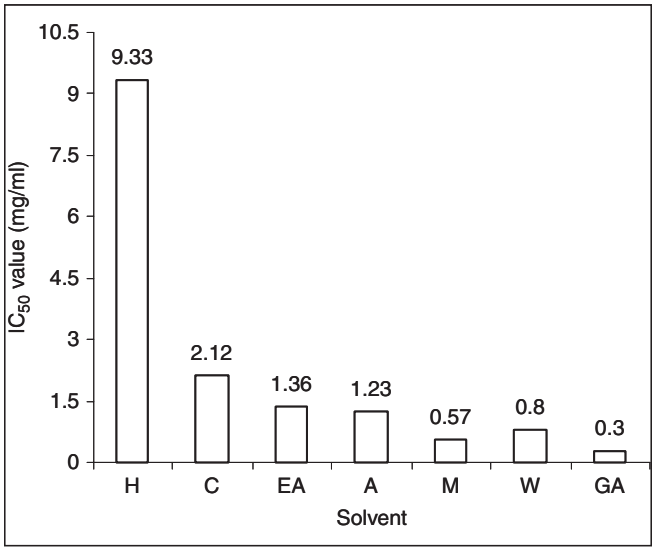

Figure 4: Comparison of HRS activity of different extracts of Ashwagandha root

with IC_{50} values of 93.98 and 124.94 µg/ml, respectively. Hexane extract was found to possess minimum SRS activity with maximum IC_{50} value of 740.46 µg/ml.

The correlation co-efficients of SRS activities with TPC and tannins were maximum (Table-2) thereby suggesting their possible involvement in this activity. Overall, the present investigation indicates that the methanolic extracts have a noticeable effect on scavenging superoxide radicals.

H_2O_2 scavenging activity

Although not a radical species, H_2O_2 is an important ROS, contributing to oxidative stress. The generation of even low levels of H_2O_2 in biological systems may be deleterious since naturally-occurring iron complexes are believed to react with H_2O_2 *in-vivo* to generate highly reactive hydroxyl radicals in a superoxide-driven 'Fenton' reaction.[46] Hence, the ability of all the extracts of Ashwagandha root to scavenge hydrogen peroxide was determined. It was noticed

Table 6: Regression equations and correlation coefficients for SRS activity of different extracts of Ashwagandha

Extract	Regression equation	Correlation coefficient (R^2)
Hexane	y = 0.0673x + 0.1667	0.9822
Chloroform	y = 0.2794x + 0.2286	0.9835
Ethyl acetate	y = 0.3263x + 1.8238	0.9905
Acetone	y = 0.2864x + 1.9893	0.9853
Methanol	y = 0.5663x – 3.2214	0.9913
Water	y = 0.3594x + 5.0952	0.9808

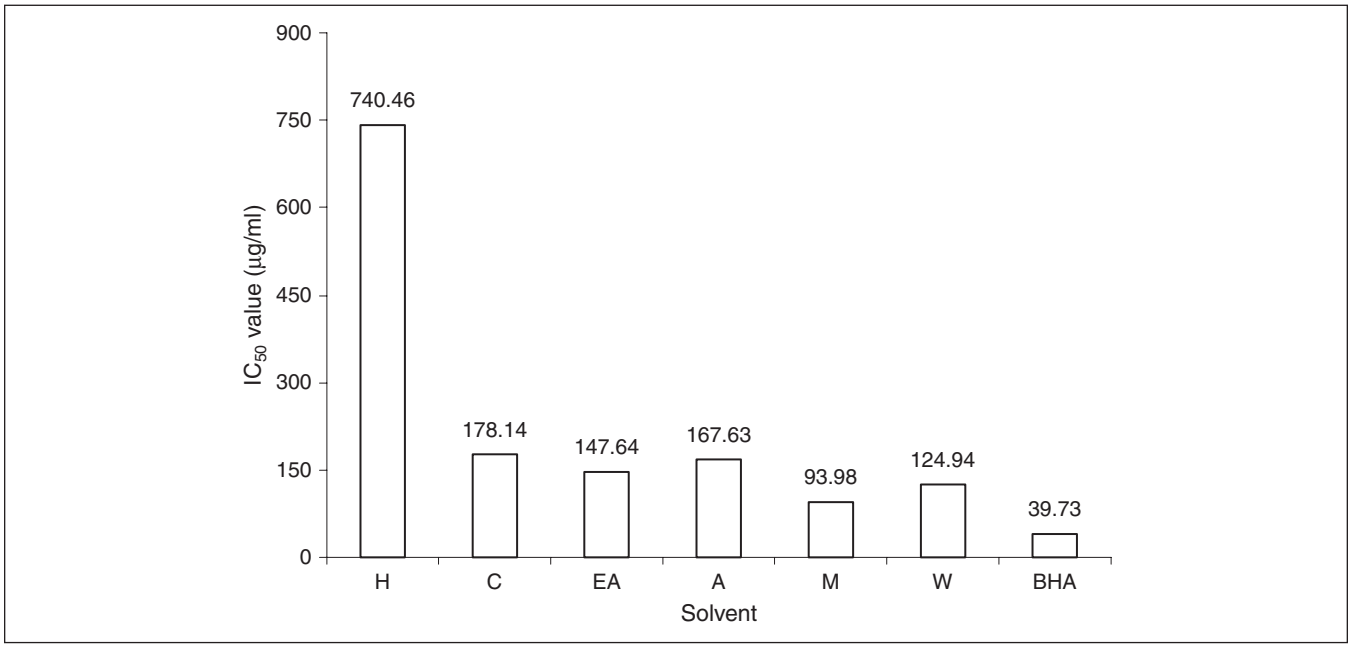

Figure 5: Comparison of SRS activity of different extracts of Ashwagandha root

that all the extracts were capable of scavenging hydrogen peroxide in dose dependent manner. The regression equations for all the extracts are presented in Table-7 while the calculated IC_{50} values are shown in Figure-6. The figure shows that methanolic extract showed good activity in depleting H_2O_2, with an IC_{50} value of 0.294 mg/ml followed by water extract (0.323 mg/ml). The correlation coefficients were derived between the H_2O_2 scavenging capacity and the presence of antioxidant components (TPC, flavonoids, flavonols and tannins). Maximum R^2 index was found with TPC which indicates that polyphenolic compounds may probably be involved in removing the H_2O_2 (Table-2).

CONCLUSION

The compounds from Ashwagandha roots were extracted in solvents of varied polarity in a sequential manner and maximum yield was recorded in methanol fraction. The methanol extract was found rich in total polyphenol contents as well as in flavonoids, flavonols and tannins. Among all the extracts, methanol extract was the most potent in terms of its radical scavenging activities tested using different assays. A correlation was established between antioxidant activity and total polyphenolic compounds present in extract. Overall, the methanolic extracts of Ashwagandha showed strong antioxidant activity, DPPH radical, superoxide anion scavenging, hydrogen peroxide scavenging and metal chelating activities which could be associated with its high medicinal value.

Table 7: Regression equations and correlation coefficients for hydrogen peroxide scavenging activity of different extracts of Ashwagandha

Extract	Regression equation	Correlation coefficient (R^2)
Hexane	y = 7.7835x – 1.51	0.9888
Chloroform	y = 12.319x + 2.5829	0.9808
Ethyl acetate	y = 0.0194x + 1.52	0.9893
Acetone	y = 0.0213x + 4.6071	0.9816
Methanol	y = 0.1438x + 7.6676	0.9477
Water	y = 0.1374x + 5.61	0.9615

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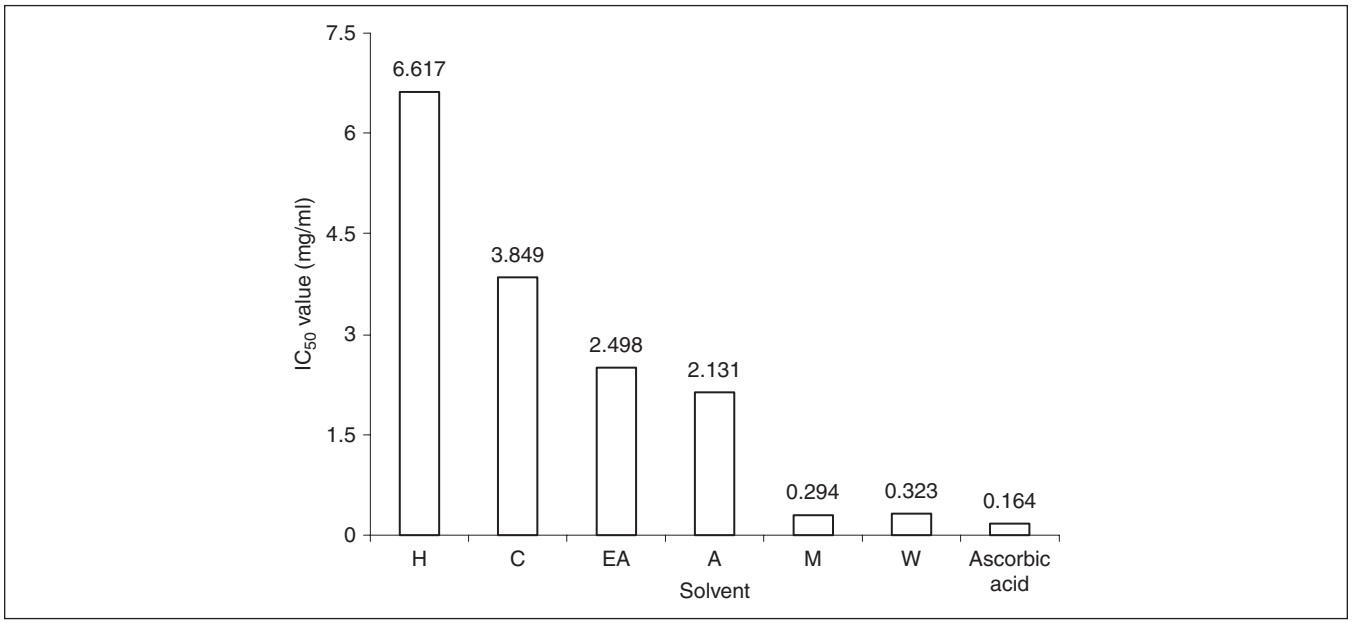

Figure 6: Comparison of hydrogen peroxide scavenging activity of different extracts of Ashwagandha root

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Antioxidant Potential of *Tinospora cordifolia* Extracts and their Protective Effect on Oxidation of Biomolecules

Ilaiyaraja N* and Farhath Khanum

Biochemistry and Nutrition Division, Defence Food Research Laboratory (DFRL), Siddhartha Nagar, Mysore-570011, India

ABSTRACT

Tinospora cordifilia is a medicinal plant, commonly known as guduchi or amritha. In the present study, the antioxidant potential of solvent extracts of leaf and stem were evaluated by various *in vitro* methods. Scavenging effects on DPPH, ABTS radical, hydroxyl radical and ferric reducing antioxidant power (FRAP) were found to be highest in methanolic extract of leaf and ethyl acetate extract of stem compared to all other extracts. These extracts also exhibited significant protection against radical induced protein (BSA) oxidation and plasmid DNA damage (pBR322). The extracts were further evaluated for their inhibitory properties on AAPH (2, 2′-azo (2-amidinopropane) dihydrochloride induced *ex vivo* oxidative stress in rat liver homogenates. The results again showed the potent antioxidant nature of methanolic extract of leaf and ethyl acetate extract of stem with respect to inhibition of lipid and protein oxidation. Overall, stem extracts showed to be the more effective antioxidant source than the leaf extracts with regard to all the radical scavenging activities. These protective properties of the extracts could be directly attributed to the presence of phytochemicals such as polyphenols, tannins etc. In conclusion, our results demonstrate the potential antioxidant activities of guduchi leaf as well as stem and therefore, it can be used as a source of antioxidant for health benefits through dietary supplementations.

Key words: *Tinospora cordifolia*; Polphenols; Tannins; Antioxidant activities; Protein oxidation; Lipid oxidation; DNA damage; Oxidative stress.

INTRODUCTION

Increasing evidence suggests that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are implicated in several degenerative diseases like cancer, asthma, arthritis, and cardiovascular problems.[1] Production of reactive oxidants such as superoxide, hydroxyl radicals and hydrogen peroxide in living cells is an inevitable process of normal oxygen metabolism. Mechanism responsible for the radical mediated injuries to cells and tissues mainly include lipid peroxidation, oxidative DNA damage and protein oxidation.[2] As a result, investigations on these biomolecules oxidation and their detrimental effects have been in focus for many years. Despite naturally occurring bodily antioxidant systems (enzymes and antioxidant nutrients) that are able to control the free radical mediated oxidative damage, its continuous exposure for a long time may lead to irreversible oxidative damage. Therefore, antioxidants particularly from dietary sources may have a great relevance in the prevention and therapeutics of such diseases for being safer and more effective in the context of their efficiency and non-toxicity than the synthetic antioxidants.[3-4]

Tinospora coridifolia is a well known medicinal plant and is widely used in folk medicine/ ayurvedic system of medicine. It is a large glabrous, succulent, climbing shrub belonging to the family of menispermaceae. Leaf, stem and roots of this plant have been shown to posses various therapeutic purposes. A range of pharmacological properties have been reported including immunomodulatory,[5-6] hypoglycemic,[7] anti-hepatotoxic,[8-9] antistress,[10] anti-inflammatory,[11] gastroprotective,[12] antioxidant,[13] radioprotective effects,[14] and memory enhancing properties.[15] Despite its long usage as testified in traditional folklore, the biological properties of various plant parts of *T. coridifilia* on free-radical scavenging ability and its effect on biomolecule oxidation are scanty. Hence, in the present study, the antioxidant properties of *T. coridifolia* leaf and stem were evaluated under *in vitro* and *ex vivo* conditions.

Address for correspondence:
E-mail: nilaiyaraja@gmail.com

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MATERIALS AND METHODS

Folin-Ciocalteu reagent (FCR), Na_2CO_3, were purchased from Sisco Research Laboratory (SRL, Mumbai). Gallic acid, $FeCl_2$, BHT, Ferric cyanide and EDTA were procured from E-Merck, Mumbai, India. DPPH was purchased from Hi-Media. BSA, pBR322 plasmid were purchased from Genei, Bangalore. Agarose and ethidium bromide were purchased from Sigma-Aldrich (St.Louis.MO). All other reagents were of analytical grade.

Preparation of extracts

The leaves and stems of *T. coridifolia* were procured from local market and dried at 50°C in a hot air oven and then powdered. 200 g powder was used for the sequential extraction using different solvent systems with the increasing polarity viz hexane, chloroform, ethyl acetate, acetone and methanol which were used (1:10 ratio) in an orbital shaker for 12 hours. Extracts were filtered and dried by flash evaporation/ lyophilization as per the requirements. Finally, the crude extracts were stored in a deep freezer (–20° C) until further use.

Total polyphenols and Tannins

Total polyphenol content of the extract was determined using Folin-Ciocalteu reagent.[16] Gallic acid was used a standard compound and amount of total polyphenols content was expressed as mg gallic acid equivalent per mg extract (mg GAE/mg). Folin-Denis method was used for estimating tannin content of the extracts[17] and tannic acid was used as a standard compound.

In vitro Antioxidant activity

DPPH(1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of the extract was determined as described by Braca *et al*.[18] ABTS (2, 2'-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) radical discoloration assay was performed as per Re *et al*.[19] The antioxidant capacity of the extract was expressed as% inhibition and IC_{50} value was calculated from regression analysis. Ferric reducing antioxidant power Assay (FRAP) was performed according to Benzie and Strain with slight modifications[20] and results were expressed in ferrous sulphate equivalent. Hydroxyl radical scavenging activity assay was performed following the method of Halliwell *et al* [21] using deoxyribose.

Protective effect on oxidation of biomolecules *in vitro*

a) Inhibition of protein oxidation

Protein oxidation was assayed as described by Kwon *et al* [22] with minor modifications. Oxidation of BSA (5 µg) in phosphate buffer was initiated by 20 mM AAPH and the inhibitory properties of extracts were measured at a fixed concentration (1 mg/ml). After incubation for 2 hours at 37°C, 0.02% BHT was added to prevent the formation of further peroxyl radical. The samples were then analysed by normal SDS-PAGE electrophoresis.

b) Inhibition of oxidative DNA damage

Conversion of the supercoiled form of plasmid DNA to open circular and further linear form has been used as an index of DNA damage. DNA strand breakage assay was performed using pBR322 plasmid DNA as per the method described by Lee *et al*.[23] A mixture of 10 µl of fixed concentration of extracts (1 mg/ml) and plasmid DNA (0.5 µg) was incubated at room temperature followed by the addition of AAPH (10 mM). The final volume of the mixture was made up to 20 µl and incubated for 30 min at 37°C. The DNA samples was electrophoresed on 1% agarose gel and band intensities were analyzed using Easy win 32 software from Herolab (Germany).

Protective effect on biomolecule oxidation *ex vivo*

Male Wistar albino rats weighing 200-220 g were housed under conventional conditions and were allowed free access to food and water, *ad libitum*. All experiments were carried out according to guidelines for the care and use of experimental animals approved by Institutional Animal Ethics Committee. The rats were anaesthetized using ether, their abdomen was opened and their liver was quickly removed. Liver was homogenized (10% w/v) in sodium phosphate buffer (pH, 7.4). Homogenate was then centrifuged at 5000 g for 15 min at 4° C. The protein concentration of the supernatant was determined by the total protein kit (Agappe diagnostics, India). Oxidative stress was induced as described previously[24] with a peroxyl radical inducer (AAPH). The reaction mixture was composed of liver homogenate (15 mg protein), 40 mm AAPH and a fixed concentration of various solvent extract of *Tinospora* stem and leaf (2 mg). The reaction mixture was incubated at 37° C for 2 hours and further analysed as below.

a) Inhibition of lipid peroxidation

The extent of lipid peroxidation of the liver homogenate was evaluated by measuring the product of thiobarbituric acid reactive substances (TBARS). After incubation, each reaction was terminated by adding 2% BHT followed by addition of 1 ml of TCA (20% w/v) to the mixture. After centrifugation at 3000 g for 15 min, the supernatant was incubated with 1 ml of thiobarbituric acid (TBA, 0.67%) at 100°C for 15 min. The color intensity of the TBARS/ TBA complex was measure at 532 nm. The amount of TBARS formed was calculated using absorbance coefficient of $1.56 \times 105\ cm^{-1}\ M^{-1}$. The data was expressed in terms of percentage inhibition.[25]

b) Inhibition of protein carbonyl formation

Protein carbonyl content of the rat liver homogenate in the presence and absence of various extracts was evaluated

by the method as described by Reznick and Packer.[26] One ml of 10 mM DNPH in 2N Hcl was added to the reaction mixture (2mg protein) and samples were incubated for 1 h at room temperature. Then, 1 ml of trichloroacetic acid, 10% was added to each reaction mixture and centrifuged at 3000 g for 10 min. the protein pellet was washed three times with 2 ml of ethanol/ethyl acetate (1:1,v/v) and dissolved in 1 ml of guanidine hydrochloride (6M, pH 2.3) and incubated for 10 min at 37°C. The carbonyl content was calculated based on the molar extinction coefficient of DNPH ($\varepsilon = 2.2 \times 10^4\ cm^{-1}\ M^{-1}$).The data were expressed in terms of percentage inhibition.

Statistical analysis

All the data were expressed as mean ± standard deviation. IC_{50} values were calculated using regression equation in excel programme. Statistical analysis was performed using student't' test. The *p* values less than 0.05 were considered as significant different.

RESULT AND DISCUSSION

In the present study various solvents namely hexane, chloroform, ethyl acetate, acetone, methanol and water were used sequentially for extracting antioxidant compounds from leaf and stem of *T. coridifolia*. Among the various leaf extracts obtained, water extract had highest amount of extractable compounds (17.0 ± 1.24%) whereas in case of stem, methanol extract had highest amount extractable compounds (6.0 ± 0.3%) as compared to other extracts (Table 1).

Total polyphenols and tannin content

It is well-known that plant phenolics, in general, are highly effective free radical scavengers and antioxidants.[27] Consequently the antioxidant activities of plant are often explained with respect to their total phenolics and tannin content. These natural antioxidants not only protect lipids from oxidation, but may also provide health benefits associated with preventing damage due to biological degeneration. As revealed by our data (Table 1), the total polyphenol was preferentially extracted in methanol extract of leaf (52.17 ± 0.52 mg/g extract) than other solvents and over all, the order of phenolic content was methanol > acetone > chloroform > water > ethylacetate > hexane. On the other hand in stem, ethyl acetate extracted highest amount of polyphenol (60.93 ± 03.21 mg GAE/g extract) and other extracts were in the decreasing order of ethyl acetate > chloroform > acetone > methanol > water > hexane. Similarly the tannin content also showed to be highest in methanolic extract of leaf (36.93 ± 1.52 mg/g extract) followed by acetone, chloroform, ethylacetae, water and hexane in the decreasing order. With respect to stem extracts, the ethylacetate extract had high amount of tannins (40.57 ± 0.23 mg/g extract) followed by acetone, chloroform, methanol, water and hexane in the decreasing order. Thus, methanolic extract of leaf and ethyl acetate extract of stem contained high levels of total polyphenols and tannins, which may account for its impressive antioxidant activity.

Antioxidant activities

a) DPPH and ABTS radical scavenging activity

DPPH and ABTS radical scavenging activities of leaf and stem extracts were evaluated (Table 2). The IC_{50} value was

Table1: Yield percentage of extractable compounds, total polyphenol and tannin content of various solvents extracts from leaf and stem of *T. coridifolia*

Solvent extracts	Extractable compounds (%)		Total Polyphenol mg GAE/g extract		Tannin mg/g extract	
	Leaf	Stem	Leaf	Stem	Leaf	Stem
Hexane	3.8 ± 0.2	0.8 ± 0.06	7.62 ± 0.16	5.45 ± 0.23	4.10 ± 0.20	0.88 ± 0.13
Chloroform	5.0 ± 0.4	2.2 ± 0.2	20.93 ± 0.66	41.51 ± 0.38	17.00 ± 0.18	28.27 ± 0.09
Ethyl acetate	1.2 ± 0.31	0.4 ± 0.01	17.55 ± 0.21	60.93 ± 3.21	12.05 ± 0.48	40.57 ± 0.23
Acetone	0.8 ± 0.07	0.6 ± 0.02	46.35 ± 4.47	36.16 ± .044	33.54 ± 0.20	29.04 ± 0.12
Methanol	13.6 ± 0.97	6.0 ± 0.3	52.17 ± 0.52	33.93 ± 1.04	36.93 ± 1.52	25.92 ± 0.10
Water	17.0 ± 1.24	5.6 ± 0.42	18.67 ± 0.58	15.55 ± 0.05	15.69 ± 0.07	12.84 ± 0.07

Table 2: DPPH and ABTS radical scavenging activity of sequential extracts from *T. coridifolia*

Solvent extracts	DPPH radical scavenging activity		ABTS radical scavenging activity	
	Leaf (IC_{50}) mg	Stem (IC_{50}) mg	Leaf (IC_{50}) µg	Stem (IC_{50}) µg
Hexane	1.62 ± 0.05	3.95 ± 0.11	284 ± 11	695 ± 23
Chloroform	1.11 ± 0.04	0.64 ± 0.02	169 ± 09	62 ± 04
Ethyl acetate	1.58 ± 0.08	0.60 ± 0.02	252 ± 12	57 ± 03
Acetone	0.71 ± 0.02	0.79 ± 0.04	105 ± 08	97 ± 06
Methanol	0.54 ± 0.01	0.74 ± 0.03	95 ± 05	107 ± 10
Water	1.22 ± 0.06	1.79 ± 0.08	143 ± 08	298 ± 13

calculated and in general, the samples with the low IC_{50} are potent scavenger than the samples with high IC_{50} value. Methanolic extract was found to be the most effective radical scavenger of DPPH radical with the least IC_{50} value (0.54 ± 0.01 mg/ml) among all the leaf extracts followed by acetone, chloroform, water, ethyl acetate and hexane extracts. Ethyl acetate extract was found to be the most effective radical scavenger with the least IC_{50} value (0.54 ± 0.01mg/ml) among all other stem extracts followed by chloroform, methanol, acetone, water and hexane extracts. IC_{50} value for the BHA standard was at 9.77 ± 0.44 µg/ml.

The results of ABTS radical scavenging activity for leaf and stem extracts also showed that the methanol extract (leaf) was the most potent radical scavenger with the lowest IC_{50} of 95 µg/ml followed by acetone > water > chloroform > ethylacetate > hexane. Ethylacetate (stem) was the most potent ABTS radical scavenger with the lowest IC_{50} of 57 µg/ml followed by chloroform > acetone > methanol > water > hexane. IC_{50} value for the vitamin C standard was at 25.26 ± 0.32 µg/ml. Since phenolic compounds have been studied extensively earlier as important contributors to the antioxidant properties,[28] the highest radical scavenging ability of the potent extracts can be directly correlated to the presence of high content of polyphenols as well as tannins. The hydrogen donating ability of these compounds is responsible for their effective antioxidant property and used for protecting against cellular oxidative damage.

b) Hydroxyl radical scavenging activity

Hydroxyl radicals are most reactive ROS, capable of attacking most of the biological substrates.[29] The prevention of such deleterious effect is very necessary in terms of both human health and the shelf-life of food and pharmaceuticals. So, it is considered important to assess the protective ability of the sample extract against $OH^{\cdot}$ radicals. In the $OH^{\cdot}$ radical - mediated 2-deoxy-D-ribose degradation assay, radicals are generated by fenton chemistry using EDTA, Fe^{3+} ions, ascorbic acid and H_2O_2. The $OH^{\cdot}$ radicals degrade the 2- deoxy-D- ribose molecule into 2-thiobarbituric acid reactive substances (TBARS), which can be quantified spectrophotometrically. The *T.coridifolia* extract (1 mg/ml) added to the reaction mixture removed the radical from the sugar and prevented it from degradation and the results are shown in Figure 1. Amongst leaf extracts, the radical scavenging activity decreased in the order of methanol > acetone > chloroform > water > ethyl acetate > hexane and amongst stem extracts, the activity decreased in the order of ethylacetate > acetone > chloroform > methanol > water > hexane. Our study shows that methanol extract (88.44%) and ethyl acetate extracts (94.73%) were the most effective $OH^{\cdot}$ radical scavengers in leaf and stem respectively.

c) Ferric reducing antioxidant power (FRAP)

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity.[30] Higher absorbance indicates higher reducing/antioxidant power of the plant samples. Figure 2 clearly shows that methanol extract is the most potent reducing agent among leaf extracts (8.97 ± 0.11 µg ferrous sulphate equivalent) and ethyl acetate extract is the most potent reducing agent among the stem extracts (16.87 ± 0.07 µg ferrous sulphate equivalent) followed by other extracts at fixed sample concentration of 0.1 mg/ml. The antioxidant activity of typical antioxidants has been attributed by various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging.[31] The data on the ferric reducing power for the extracts suggest that it contributes significantly toward the observed antioxidant effect.

Protective effect on Biomolecules oxidation *in vitro*

a) Inhibition of Protein oxidation

Accumulation of macromolecular oxidative damage has been reported as a fundamental cause in many pathological

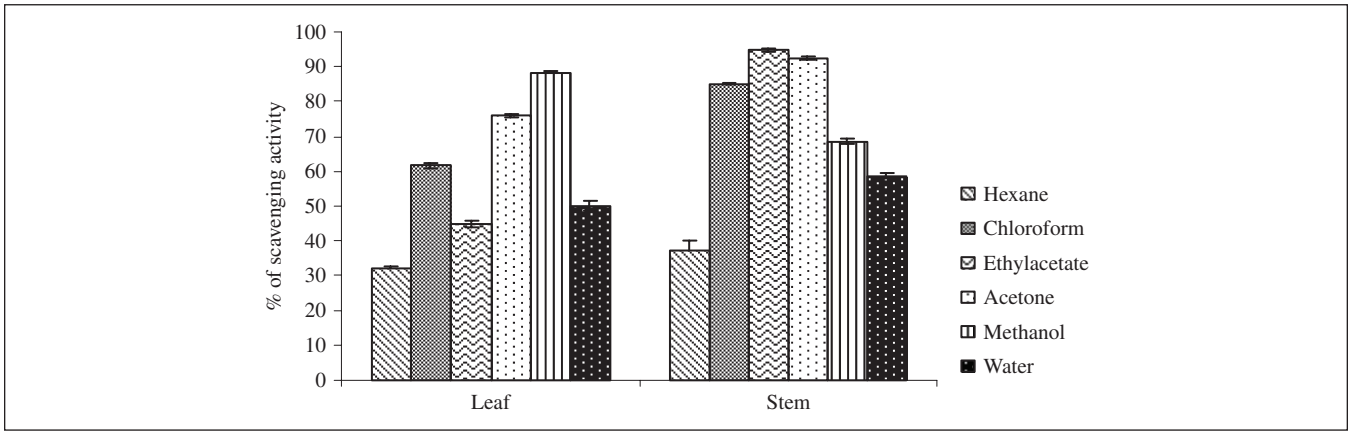

Figure 1: Hydroxyl radical scavenging activities of *T. coridifolia* extracts

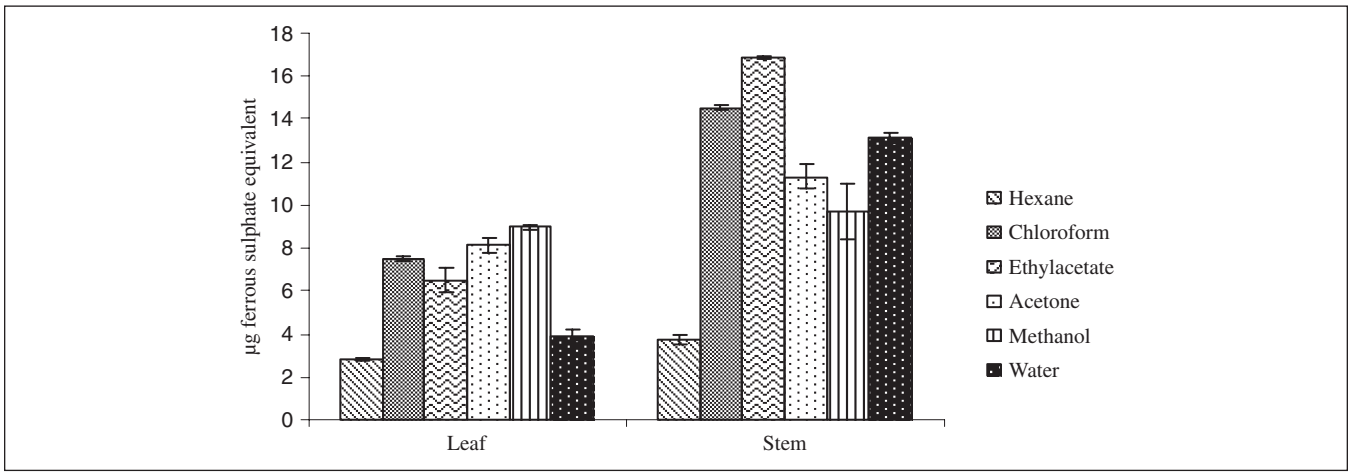

Figure 2: Ferric reducing antioxidant power (FRAP) of *T. coridifolia* extracts

conditions. Cellular proteins are one of the vulnerable targets for oxidation caused by reactive oxygen species. AAPH is a water soluble initiator, which decomposes at physiological temperature producing alkyl peroxyl radicals with oxygen to initiate the protein oxidation.[32] The protection by the *T.coridifolia* extract against protein oxidative damage was determined by the oxidation of BSA initiated by AAPH. Figure 3 shows that two hours after the incubation, the BSA was completely degraded by 20 mM AAPH in positive control as studied by SDS-PAGE electrophoresis (lane 2). Among all the leaf extracts, only methanol (lane 7) and ethyl acetate (lane 6) extract prevented the BSA oxidation significantly by 55 and 50% respectively compared to negative BSA control (lane 1) at the concentration of 1 mg/ml . Though all the stem extracts showed a significant protective effect, ethyl acetate was found to be the most potent extracts which completely prevented the oxidation (lane 8) whereas other extracts showed up to 50% protection only. Comparatively, the protective capacity of stem extracts was found to be more than leaf extracts might be due to the higher radical scavenging activity of the former.

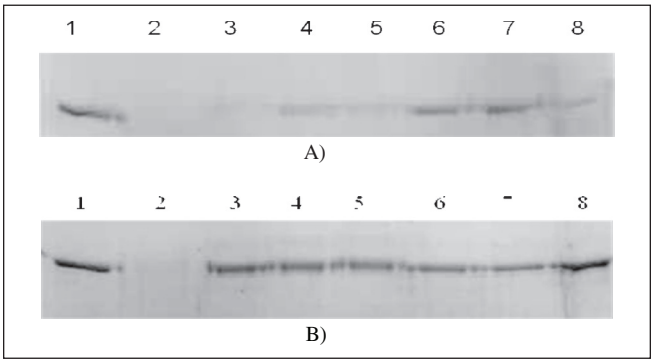

Figure 3: The protection of *T. coridifolia* extracts (1 mg/ml) on AAPH (40 mM) induced oxidative fragmentation of BSA. A) Lane 1, BSA alone; lane 2, BSA + AAPH (positive control); lane 3-8, BSA + AAPH + leaf extracts (hexane, chloroform, ethyl acetate, acetone, methanol and water). B) Lane 1-BSA alone; lane 2-BSA + AAPH (positive control); lane 3-8 BSA + AAPH + stem extracts (hexane, chloroform, acetone, methanol, water, ethyl acetate).

b) Inhibition of DNA damage

Oxidative modification of DNA has been suggested to contribute to aging and various diseases including cancer and chronic inflammation.[33] Therefore, the protective effect of extracts on oxidative DNA strand breakage was evaluated with pBR322 plasmid DNA. As shown in Figure 4 the plasmid DNA was mainly of the supercoild form (bottom band) and open circular form (top band) in the absence of AAPH (lane 1). With addition of 10 mM AAPH, the supercoild form decreased and converted into the open circular form (lane 2). Addition of extract significantly ($p < 0.05$) inhibited the formation of open circular form compared to positive control. The % of DNA protection offered by the leaf extracts (1 mg/ml) was in the decreasing order of methanol (79%) > ethylacetate (73%) > chloroform (71%) > acetone (68%) > hexane (50%) and water extract did not show any protection. With regard to stem, acetone and ethylacetate extracts were the most effective with 96% inhibition followed by chloroform (90%), methanol (81%), water (74%) and hexane (48%) extracts. Our results shows that methonolic extract of leaf, and acetone and ethylacetate extract of stem are the potent agents in preventing the oxidative DNA damage.

Protective effect on biomolecule oxidation *ex vivo*

a) Inhibition of lipid peroxidation

The AAPH model is a well-established system for investigating *in vivo* and *in vitro* lipid peroxidation. In this study, the potential of each of the extracts to inhibit lipid peroxidation in the rat liver homogenate induced by the AAPH was measured. The addition of AAPH to the liver homogenate for 2 hrs significantly increased the extent of TBARS formation relative to the control sample. Induction

of oxidative stress in rat liver homogenate uniformly resulted in an increase in lipid peroxidation levels. However, treatment with the plant extracts significantly decreased TBARS content. Figure 5 clearly demonstrates that the inhibition of lipid peroxidation in leaf extracts (2 mg/ml) was most effective in methanolic extract (69.19%) followed by acetone > ethylacetate > water > chloroform > hexane while among stem extracts, ethylacetate extract (80.23%) was the most effective one followed by acetone > chloroform > methanol > hexane > water extracts. As a positive control, catechin (50 μg/ml) showed high inhibitory effect (91.23%). Consequently, it can be suggested that plant extracts, may be effective in preventing lipid oxidation which is believed to occur during oxidation processes.

b) Inhibition of protein carbonyl formation

Proteins are known to be damaged by ROS directly and to be targets of secondary modifications by aldehydic products of lipid peroxidation or ascorbate autooxidation. All these processes can be collectively result in carbonyl modification of protein (PCO). The assessment of PCO is a widely-used marker for oxidative protein modification and it is reported to be a sensitive and early marker of oxidative stress to tissues as compared with lipid peroxidation.[34] Figure 6 shows the % of inhibition by the extracts (2 mg/ml) against the oxidation of proteins which is measured in terms of protein carbonyl content. Among leaf extracts, the methanol extract showed the highest inhibition (70.25%) followed by acetone, ethylacetate, water, chloroform and hexane in the decreasing order. Among stem extracts, ethylacetate showed

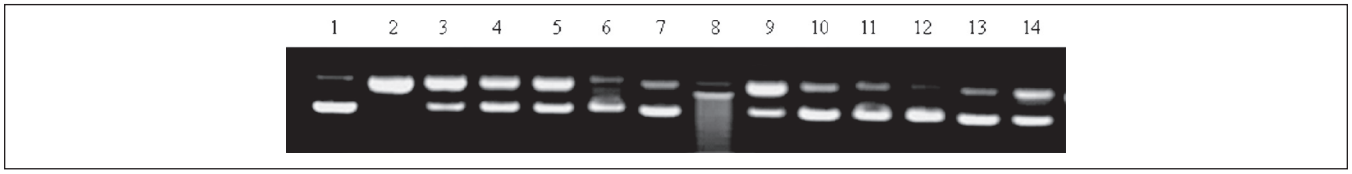

Figure 4: The protection of *T. coridifolia* extracts (1 mg/ml) on 10 mM AAPH induced pBR322 plasmid DNA strand breakage. Lane 1-plasmid DNA alone; lane 2-plasmid DNA + AAPH (positive control); lane 3-8, pBR322 plasmid DNA + AAPH + leaf extract (hexane, chloroform, ethyl acetate, acetone, methanol and water); lane 9-14, plasmid DNA + AAPH + stem extract (hexane, chloroform, ethyl acetate, acetone, methanol and water).

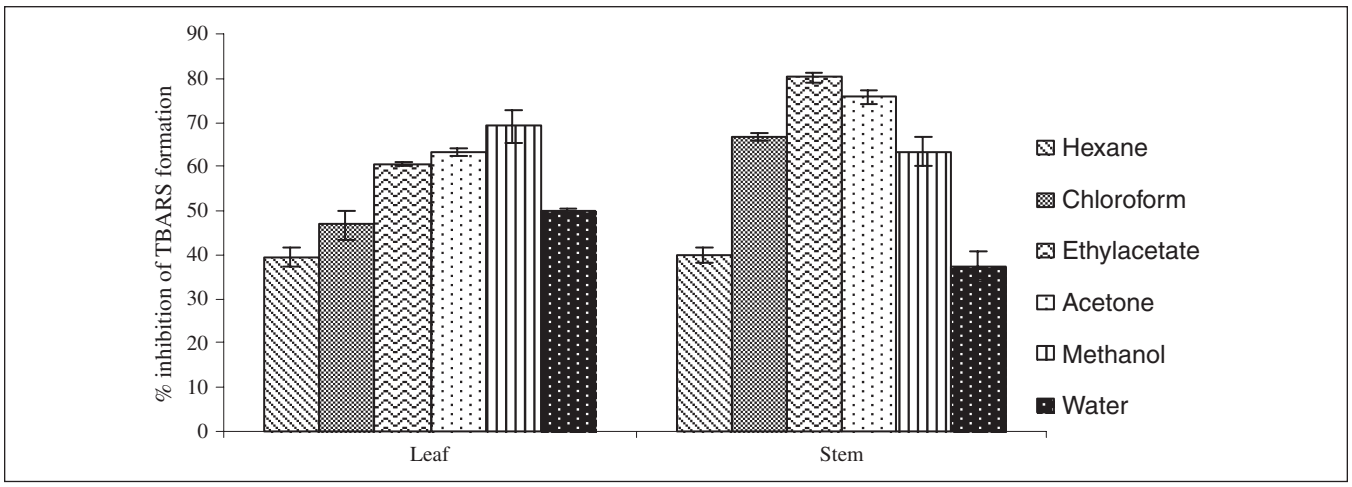

Figure 5: Inhibitory effect of *T. coridifolia* against AAPH reagent induced lipid peroxidation.

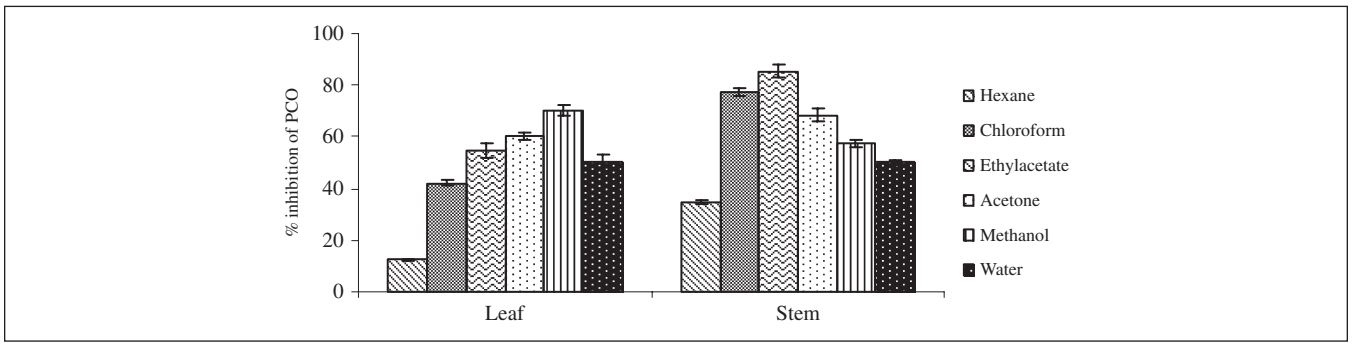

Figure 6: Inhibitory effect of leaf extract of *T. coridifolia* on AAPH reagent induced PCO formation.

highest inhibition (85.45%) followed by chloroform, acetone, methanol, water and hexane. Catechin standard (50 µg/ml) showed 96.72% inhibition against PCO formation. The inhibitory effect of extracts might operate by scavenging the peroxyl radical generated in the reaction mixture.

CONCLUSION

The results of present study indicate that the methanolic extract of leaf and ethyl acetate extract of stem are the most potent extracts compared to other solvent extracts towards various radical scavenging activities. These extracts also conferred significant protection against oxidation of biomolecules such as proteins, DNA and lipids. The radical scavenging ability of the extracts could be due to the presence of phenolic compounds. Overall, the stem extracts showed to be the more effective antioxidant source than the leaf extracts with regard to all the parameters analysed. Therefore stem of *T. coridifolia* can be considered as a potential source of natural antioxidants over the leaf for pharmaceutical use or dietary supplementations.

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Pharmacognostic studies on *Terminalia citrina* (Gaertn) Roxb. ex Fleming.

Priyanka Ingle and Arvind Dhabe

Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, 431004. (MS) India.

ABSTRACT

Terminalia citrina (Gaertn.) Roxb. ex Fleming synonym *Terminalia multiflora* Merr. is commonly known as Yellow Myrobalan and *Manahei* in Manipuri. It is highly medicinal plant which is distributed in North East India and Car Nicobar Island. Its bark is diuretic and cardio tonic and fruits are used similar to *Terminalia chebula* and used in various drug preparations, which are sometimes adulterated with other plant materials. In order to standardize the drug, detail morphological, anatomical (T.S. of bark, petiole and leaf; T.S., T.L.S. and R.L.S. of wood and maceration of bark and wood) and dermatological studies were undertaken.

T.S. of bark showed peculiarities as-bundles of fibre and patches of stone cells, compound crystals and compound starch grains. Maceration of bark showed unique branched phloem fibres, and thin and thick walled phloem parenchyma and various crystals. T.S. of bark showed Vessels in groups of 2-9. Parenchymatous rays mostly uniseriate, rarely biseriate, heterogenous with deposition of starch grains. Maceration of wood reveled the diagnostic features like-pitted xylem parenchyma, branched xylem fibres with pits, various types of vessel elements with oblique end walls with or without long beaks, pits alternate. Tracheids tubular, thick walled with small and few pits.

It was observed that *Terminalia citrina* (Gaertn.) Roxb. ex Fleming can be easily standardized on the basis of combination of these parameters which are peculiar to this species.

Key words: Standardization, *Terminalia citrina,* Anatomy.

INTRODUCTION

Terminalia citrina (Gaertn) Roxb. ex Fleming synonyms *Combretum arboreum* Miq., *Embryogonia arborea* Teijsm. & Binn. ex Miq., *Myrobalanus citrina* Gaertn., *Terminalia curtisii* Ridi., *Terminalia multiflora* Merr., *Terminalia teysmannii* K. & V.[6] It is tall tree belongs to family Combretaceae, which popularly known as Yellow Myrobalan, *Manahei* in Manipuri and *Monalu* in Andamani.[7-8] It is highly medicinal plant which is endemic to North East India and Car Nicobar Island.[3-7] Its bark is diuretic, cardio tonic and fruits are used similar to *Terminalia chebula,* used in various drug preparations.[5-8] Its wood is also used as timber, making furniture and to yield dye. Because of excessive exploitation and depletion of tree population, it has been adulterated with other plant materials. In order to standardize and to detect adulteration in this crude drug, an attempt has been made to develop the anatomical parameters of bark, wood and leaves.

Address for correspondence:
E-mail: priyaingalkar@gmail.com
E-mail: arvind@asdhabe.com

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MATERIALS AND METHODS

Plant material was collected from a tree planted at Govindaji Temple, Imphal, Manipur (Lattitude N24°52, 893"; Longitude E093°54, 829", Altitude 2518 ft).Transverse sections (T.S.) of bark, wood, petiole and leaf, tangential longitudinal section (T.L.S.) and radial longitudinal section (R.L.S.) of wood were taken by free-hand method; they were double stained and mounted permanently as per standard procedures. The tissue of bark, wood and leaf for dermatological studies were macerated by Jeffery's method. Trichomes were studied by scraping.[4] The slides were prepared temporarily. The barks were ground in mixer and grinder to fine powder, the fine powder was mixed in water and allowed it to settle down. The supernatant was decanted. This procedure was repeated for several times to remove the cells and tissues (plant debris). The white crystals were left out. These crystals were placed on slides

and observed under microscope. Their dimensions were measured with the help of standard micrometry slide and ocular eye piece.[1] Microphotographs were taken by using Sony digital camera.

RESULT AND DISCUSSIONS

Transverse section of bark showed 25-32 layers of tubular cells. Outer 10-15 layers deposited with tannin (Photo plate **1a**). Medularly rays uniseriate diposited with tannin and starch grains. Stone cells in group of 1-6, ca 20-52.5 µ in diameter (Photo plate **1b**). Parenchymatous cells showed deposition of compound crystals ca 15-95 µ and compound starch grains ca 20-52.5 µ (Photo plate **1d, e**). Some bundles of phloem fibers of 4-79 were observed in a ring, which present in group (Photo plate **1c**). Some crystals are isolated from bark powder ca15-95 µ (Photo plate **1l**).

Maceration of bark showed thick walled, spindle shaped phloem fibers measuring ca 12.5-30 × 1501-2907 µ (Photo plate **1g**). Sieve element ca 20-30 × 310-650 µ (Photo plate **1f**). Some branched fibers were also observed and it was very diagnostic character to this species (Photo plate **1h & i**). Phloem parenchyma cells thin walled ca 17.5-25 × 22.530 µ (Photo plate **1j**), some thick walled ca 30-40 × 40-90 µ (Photo plate **1k**).

Transverse section of wood showed diffuse porous nature (Photo plate **2a**). Vessels circular to oval, mostly single, rarely in groups of 2-9, ca 30-37.5 µ in diameter. Ray parenchyma continuous with deposition of starch grains. Tangential longitudinal section of wood showed row of vessel with simple, alternate pits (Photo plate **2b**). Parenchymatous rays uniseriate to biseriate measuring about 20 × 40-600 µ, with 2-25 cells per ray. Radial longitudinal section of wood showed heterogeneous ray parenchyma, cells square and rectangular with deposition of starch grains (Photo plate **2c**).

Maceration of wood showed pitted parenchyma ca 20-47.5 × 32.5-87.5 µ (Photo plate **2e & f**). Some parenchymatous cells with few pits ca 20-47.5 × 40-87.5 µ (Photo plate **2d**). Xylem fibers simple, spindle shaped, thick walled with narrow lumen, some are without pits and some are with pits (Photo plate **2g, i**), some fibers branched, this was very diagnostic character of the species, they were measured ca 20-35 × 532-1254 µ (Photo plate **2h**). Vessel elements of two types– without beak ca 30-130 × 190-470 µ (Photo plate **2j**) and beaked ca 40-120 × 190-470 µ (Photo plate **2k**), pits simple, alternate, end wall oblique. Tracheids tubular, thick walled, ca 20-40 × 250-700 µ with small pits (Photo plate **2l**).

Transverse section of petiole showed single layered epidermis, cells filled with tannin (Photo plate **3a**). Cortex

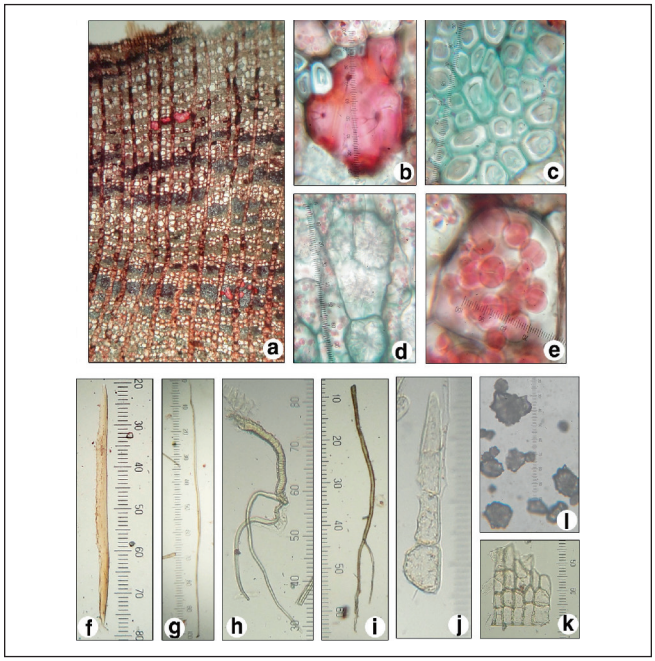

Plate 1

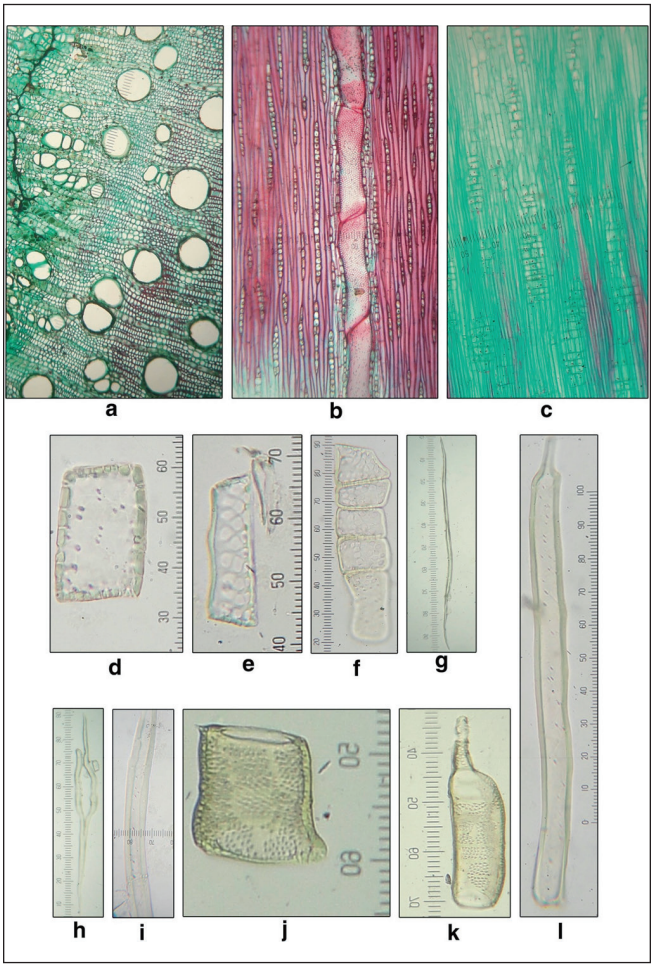

Plate 2

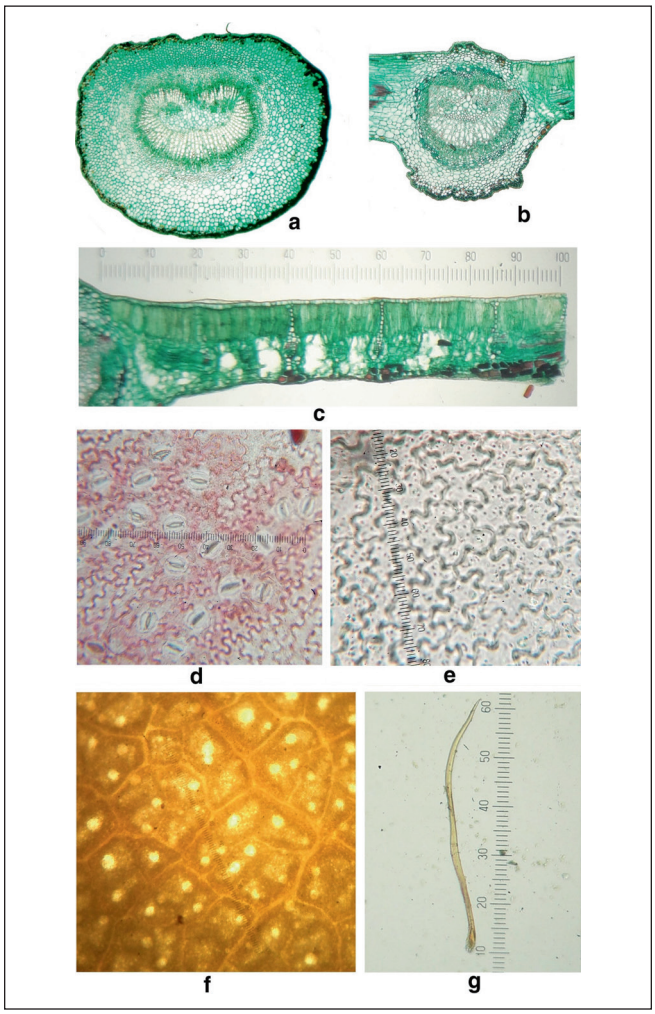

Plate 3

parenchymatous, compound crystals observed frequently. Vascular bundle conjoint, collateral and open.

Transverse section of leaf showed single layered epidermis followed by double layered palisade below which 6-8 layers spongy parenchyma were observed (Photo plate **3c**). Vascular bundle conjoint, collateral and open (Photo plate **3b**).

In the dermatology leaf showed palisade ratio 23.3, vein islet no. 9.2, veinlet termination no.11.6 (Photo plate **3f**). Stomatal index 768.6 in 1 mm^2 area. Stomata anomocytic, amphistomatic, few stomata scattered along midrib on upper surface. Upper epidermal cells ca 28.4 × 44.09 μ (Photo plate **3e**), lower epidermal cells 26.79 × 43.21 μ (Photo plate **3d**). Trichomes simple, unicellular, uniseriate ca 280.17 μ in an average, which was present on both the surfaces (Photo plate **3g**).

CONCLUSION

The above anatomical and dermatological parameters in combination were found to be diagnostic for standardization of *Terminalia citrina* (Gaertn) Roxb. ex Fleming. and also for detecting the adulteration.

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Evaluation of Brine shrimp lethality and Antimicrobial activity of *Azadirachta indica* leaf extract on some drug resistance bacteria in Bangladesh

Abdullah-Al-Emran[1], S.M Shahed[1], Farzana Ahmed[1], Sajal Kumar Saha[2], Sreedam Chandra Das[3], Sitesh Chandra Bachar[4]

[1]Department of Genetic Engineering and Biotechnology, University of Dhaka, Bangladesh, [2]Department of Clinical Pharmacy and Pharmacology, University of Dhaka, Bangladesh, [3]Department of Pharmacy, Southeast University, Dhaka, Bangladesh. [4]Department of Pharmaceutical Technology, University of Dhaka.

ABSTRACT

Azadirachta indica (Neem) is very useful traditional medicinal plant in the subcontinent. Each part of the tree has some medicinal properties. In this present studies the bioactivity and antimicrobial activity of *Azadirachta indica* leaf extract was evaluated. For bioactivity, Brine shrimp lethality test was done for *A. indica* ethanolic leaf extract and determined LC_{50} was 37.15 μg/ml indicating that the prepared extract was rich in bioactive compounds. Ethanolic leaf extract was also used to evaluate antibacterial activity and the extract exhibited significant activity against the tested bacterial strain. However, inhibitory activity was concentration and test organism dependent. Gram-positive bacteria were more sensitive to the extract compare to Gram-negative bacteria. All of the tested bacteria showed sensitivity at higher concentration (7mg/ml) but multi drug resistant bacteria *Klebsiella pneumoniae* was very sensitive even at very low concentration (2mg/ml). Further, the results depict that leaf extracts of *Azadirachta indica* could be used as a potential source of antimicrobial agents against the bacterial strains tested.

Key words: *Azadirachta indica,* Medicinal Plant, Antibacterial Activity, Agar well diffusion assay.

INTRODUCTION

The indiscriminate and improper use of antibiotics is playing a significant role in the emergence of resistant bacteria.[1] Other practices contributing towards resistance include the addition of antibiotics to the feed of livestock.[2,3] In recent years, multiple drug resistance in both human and plant pathogens has been developed due to indiscriminate use of synthetic drugs especially in the developing countries.[4] Thus, a diverse arsenal of new antibacterial agents is urgently needed to combat the diminishing efficacy of existing antibiotics.[5] One of the strong solutions of this emerging problem is the use of medicinal plant in healing this type of infection. This drives the need to screen medicinal plants for novel bioactive compounds as plant based drugs are biodegradable, safe and have fewer side effects.[6]

Address for correspondence:
E-mail: emran_geb@yahoo.com

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Bangladesh is a good source of the medicinal plants belonging to various families. ***Azadirachta indica*** (Bengali name: Neem) is a tree in the Meliaceae family. It is one of two species in the genus *Azadirachta*, and is native to India, Burma, Bangladesh, Sri Lanka, Malaysia and Pakistan, growing in tropical and semi-tropical regions. This plant investigated in Bangladesh has been reported to have a wide range of secondary metabolites including cytotoxic compounds.[7] For thousands of years the beneficial properties of *A. indica* have been recognized. Each part of the *A. indica* tree has some medicinal property and has been used as traditional medicine for household remedy against various human ailments, from antiquity.[8-13]

Although a large number of compounds have been isolated from various parts of neem, a few of them have been studied for biological activity. Sulphur-containing compounds such as cyclic trisulphide and tetrasulphide isolated from the steam distillate of fresh, matured neem leaves have antifungal activity against *Trichophyton mentagrophytes*. Different studies shows that leaf extract of this plant also has antiviral activity against Vaccinia virus, antimalarial

activity, antibacterial activity and antifungal activity against certain human fungi.[14]

Leaf extract of *Azadirachta indica* has two bioactive compounds named Rutin and Quercetin.[15] *Azadirachta indica* aqueous leaf extract significantly prevents and reverses the hepatotoxic damage induced by antitubercular drugs in rats.[16] Ethanol extract of *Azadirachta indica* leaf plays a significant role in the control of atherosclerosis and management of diabetic vascular complications.[17]

So far, in Bangladesh antimicrobial studies of leaf extract of *A. indica* against pathogenic bacteria have not been studied. On this perspective, the present work was objected to carry out scientific investigation on antimicrobial activities of ethanolic leaf extract of *A. indica* against selected drug resistance bacteria.

MATERIALS AND METHODS

Materials

Leaf part of test plant was collected from Jagannath Hall, University of Dhaka, Dhaka-1000 and was identified through national herbarium. Ethanol (Merck, Germany), Whattman filter paper, Erlenmeyer flask, measuring cylinder, and funnel, round bottom flask, simple mechanical grinder, mechanical rotator and alluminium foil were purchased from local vendors. Balance used here was made by Mettler Toledo, Solvent evaporator was supplied by J-Kem's KEM-Vap and freeze dryer used here was supplied by Millrock Technology.

Preparation of plant extract

For preparing plant extract, the collected leaf of *A. indica* was sun dried for 3 days. Then the sample was powdered using mechanical grinder. Dried leaf powder (125 gm) of *A. indica* was soaked with 250 ml ethanol in a conical flask. The flask was covered with aluminum foil and then kept on a mechanical rotator for 24 hours. Next day, the solution was filtered by whatman filter paper and the filtrate was collected. Solvent evaporator was used to evaporate ethanol in the crude preparation under reduced pressure until a gummy substance was obtained. Then approximately equal amount of water was added to the gummy substance and again the solvent was evaporated until a gummy substance was found. Then the substance on the round bottom flask was freeze-dried to prepare the powdered form of plant extract. The powdered extract was then transferred to a 50 ml Erlenmeyer flask and then weight of the powdered extract was determined using balance. 12.2 g of *A. indica* extract was obtained from 125 g of dried leaf powder. (Table 1)The prepared extract was stored in –20°C.

Brine shrimp lethality bioassay

Brine shrimp (*Artemia salina*) lethality bioassay was carried out to check the cytotoxic activity of the plant extract. The assay was done according to Meyer's process with some modification.[18] Simply, brine shrimp eggs were collected from local market and hatched with properly aerated filtered seawater for 48 hrs. After hatching, active nauplii were collected and 10 nauplii were drawn through a dropper and placed in each well of microtitre plate containing 250 µl of seawater. Then 50 µl of plant extract solution (extract dissolved in 40% ethanol) was added to make final concentration of plant extract as 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml in respective treatments. Vincristine sulphate (Gedeon Richter Ltd., Hungary) was used as positive and seawater was used as negative control. After 24 hrs, dead and live were counted under microscope. Each experiment was performed in three replicas. The percentage of mortality was then determined. Lethal Concentration 50 (LC_{50}) value was obtained from the best-fit line by plotting concentration versus percentage of mortality.

Antibiogram of microorganisms

Antibiogram of test organism was done by disc diffusion method.[19] In this process, 5 different types of antibiotic discs were used. 100 µl of inoculum was taken to each Muller Hinton agar plates using micropipette and spreaded well using sterile spreader. Then the antibiotic discs were placed accurately on the agar at appropriate distance using sterile forceps. Then the plates were inverted and incubated overnight at 37°C. Next day, the diameter of clear zone was measured and significance of zone of inhibition was determined using Kirby-Bauer method[20] of antimicrobial sensitivity testing.

Antibacterial screening

The microbial strains (Table 2) used for the experiment were collected as pure cultures. Both Gram-positive and Gram-negative bacteria were taken for the test.

Antimicrobial resistance is a worldwide problem that has deleterious long-term effects. This development of drug

Table 1: Properties of prepared plant extracts

Name of Plant	Type of Extract	Amount of Powder Taken (gm)	Extract Obtained (gm)	% of Extract Obtained	Properties of Extract
A. indica	Ethanolic leaf extract	125	12.2	9.76%	Dried amorphous power, gummy, bitter in taste

resistance surpasses the development of new drugs. Poverty has been cited as a major force driving the development of antimicrobial resistance by the World Health Organization (WHO). In developing countries, factors such as inadequate access to effective drugs, unregulated dispensing and manufacture of antimicrobials, and shortened antimicrobial therapy because of cost are contributing to the development of multidrug-resistant organisms. Many developing countries allow the dispensation of antibiotics without a prescription; this can lead to self-medication and dispensation of drugs by untrained people. In a study from Bangladesh it was observed that, 92% of medications dispensed by pharmacies were dispensed without a prescription[20] International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) conducted a study at Matlab rural surveillance area in Bangladesh to identify practices which might affect microbial drug resistance and showed that forty-eight per cent of the antibiotic tablets or capsules for persons aged 15 years or more were purchased in quantities which represented less than a single day's dose and rarely was a full course of therapy purchased at one time. It was also observed that the rate of tetracycline purchase for young children equaled the rate for older children and young adults. These practices have probably not improved health and may have promoted the emergence and persistence of drug-resistant micro-organisms.[21]

E. coli was isolated from urinary tract infected patients of Bangladesh and it was observed that isolated strain was sensitive to doxicycline ad gentamycin but resistance to amoxicillin, tetracycline, cloxacillin, cotrimazole and cephalexin.[22] Penicillin resistant pneumococcus was reported worldwide after 1974 and it was also reported in Bangladesh.[21]

Inoculum was developed according to 0.5 McFarland standard, that is, 1.5×10^8 cfu/ml, by taking OD at 600 nm using spectrophotometer. Simply, 50 µl of 1% $BaCl_2$ was mixed well with 9.95 ml H_2SO_4 and suitable three single colonies of microorganism were picked up from subculture and were suspended with normal saline (0.9% NaCl

Table 2: List of test Gram-positive and Gram-negative bacteria

Gram-positive Bacteria	*Staphylococcus aureus, Streptococcus faecalis*
Gram-negative Bacteria	*Coliform spp., Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,*

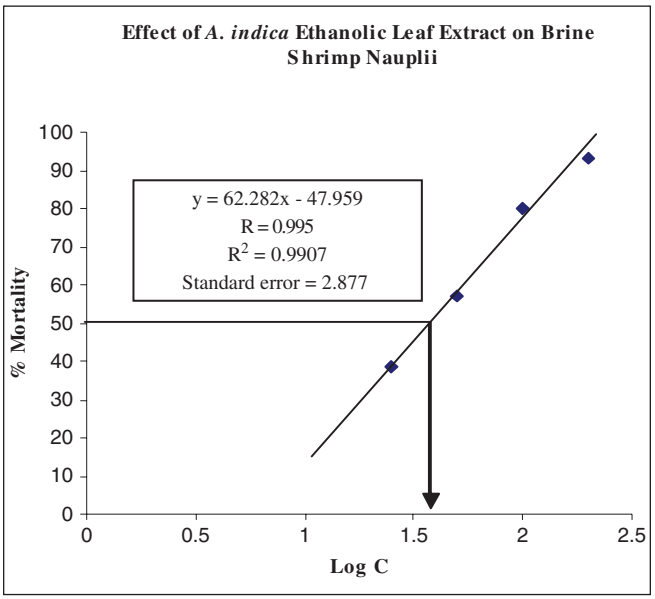

Figure 1: Determination of lethal concentration 50 (LC_{50}) of *Azadirachta indica*. A straight line obtained by plotting percentage of mortality of brine shrimps nauplii against the logarithm of the concentration of leaf extract (from Table 3). From the graph, log LC_{50} was obtained at 50% mortality. LC_{50} value was obtained by inversing the log LC_{50} value. LC_{50} value obtained for *A. indica* extract was 37.15 µg/ml.

Table 3: Effect of *A. indica* ethanolic leaf extract on brine shrimp nauplii

Conc. (µg/ml)	*Log C*	Nauplii		% Mortality	Log LC_{50} (µg/ml)	LC_{50} (µg/ml)
		Total	Dead			
Blank	–	10	0	0.00		
Solvent	–	10	0	0.00		
25	1.398	13	5	38.46	1.57	37.15
50	1.699	14	8	57.14		
100	2.0	10	8	80.00		
200	2.301	15	14	93.33		

Table 4: Effect of Vincristine sulphate on brine shrimp nauplii

Conc. (µg/ml)	*Log C*	% Mortality	Log LC_{50} (µg/ml)	LC_{50} (µg/ml)
Blank	–	0.00		
Solvent	–	0.00		
1.25	0.096	70.00	–0.539	0.288
2.5	0.397	83.33		
5	0.698	90.00		

solution). Then absorbance of microbial suspension was adjusted to 0.5 McFarland standard by adding proper amount of normal saline to it. This inoculum was prepared every time just before placing antibiotic disc or before applying plant extract on well.

The ethanolic extract was checked for antibacterial screening by agar well diffusion method.[24] 100 µl of inoculum was taken to each Muller Hinton agar plate using micropipette and spreaded well using sterile spreader. Using sterile tips, wells were produced on the agar plate. The plant extract of 100 µl was taken from each concentration of 5 mg/ml, 10 mg/ml, 20 mg/ml, 30 mg/ml, 40 mg/ml, 50 mg/ml, 60 mg/ml, 70 mg/ml and 100 mg/ml, and were applied in each well. Also, on another plate, wells were created as same as above 100 µl 40% ethanol was applied as a negative control. Then the plates were kept upside down to allow sufficient diffusion of the materials from the wells to the surrounding agar medium and were incubated in an incubator overnight at 37°C. The antimicrobial potency of the extract was measured by its activity to prevent the growth of microbes surrounding the wells, which give clear zone of inhibition. After incubation, the antimicrobial activity of the test materials was determined by measuring the diameter of the zones of inhibition in millimeter (mm) using scale.

RESULTS

Brine shrimp lethality bioassay

The extract of *A. indica* was evaluated for brine shrimp lethality in different concentrations. Vincristine sulphate was used as positive control. All experiments were done in triplicate and the mean result was noted. The lethal concentration LC_{50} of the test samples after 24 hrs was obtained by a plot of percentage of the dead nauplii against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis (Figure 1). From the assay, LC_{50} value of the leaf extract was determined and it was 37.15 µg/ml (Table 3). The positive control was used here to check the validity of the test and LC_{50} of the control was 0.288 µg/ml (Table 4, Figure 2).

Antibacterial activity of plant extract

Here it was investigated whether the extract of ***A. indica*** could induce any antibacterial activity. For this, first of all the antibiogram of some Gram-positive strains (*Staphylococcus aureus, Streptococcus faecalis*) and Gram-negative strains (*Coliform spp., Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa)* was done. For this test, ampicillin, chloramphenicol, erythromycin, rifampicin and tetracycline were used as reference antibiotics. Sensitivity against the antibiotics of a microorganism was determined by Kirby-Bauer method, 1995. The result obtained from such test (Table 5) showed that the strains used here were found to be resistant against certain types of antibiotics. Both *Klebsiella pneumoniae and Pseudomonas aeruginosa* gram negative strains were resistant against ampicillin, erythromycin, rifampicin and tetracycline but were sensitive against chloramphenicol. *Coliform spp.* was resistant to all antibiotic except tetracycline. Again gram positive strains like *Staphylococcus aureus* was resistant to ampicillin, erythromycin but *Streptococcus faecalis* was not resistant to any tested antibiotics.

The antimicrobial activity of *A. indica* ethanolic leaf extract was examined in the present study. The results

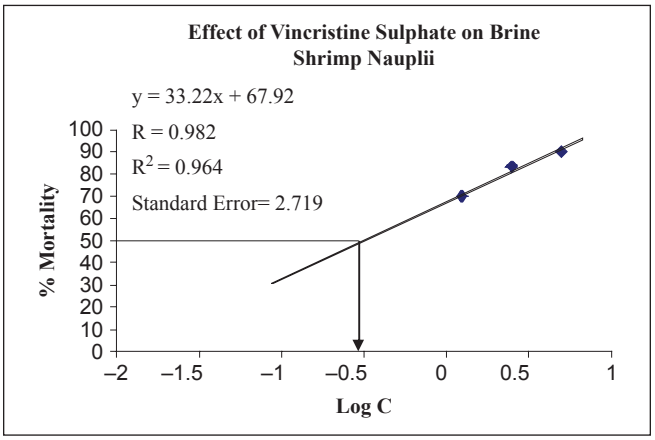

Figure 2: Regression analysis of effect of vincristine sulphate on brine shrimp nauplii. Brine shrimp nauplii were treated with different concentrations of vincristine sulphate as indicated and after 24 hours number of live and dead nauplii was counted and percentage of mortality was calculated. A straight line obtained by plotting percentage of the shrimps killed against the logarithm of the concentrations.

Table 5: Summary of Antibiogram of selected strains

Name of Microorganism		Amp. (75 µg)	Chlora (30 µg)	Ery. (15 µg)	Rif. (5 µg)	Tet. (30 µg)
Gram-Positive	*Staphylococcus aureus*	R	S	R	I	I
	Streptococcus faecalis	I	S	I	I	S
Gram-Negative	*Coliform spp.*	R	R	R	R	I
	Escherichia coli	R	S	I	S	S
	Klebsiella pneumoniae	R	S	R	R	R
	Pseudomonas aeruginosa	R	S	R	R	R

*R: Resistant, S: Sensitive and I: Intermediate. Amp.: Ampicillin, Chlora.: Chloramphenicol, Ery.: Erythromycin, Rif.: Rifampicin and Tet.: Tetracycline. Content is given as per disc of antibiotic.

Table 6: Antibacterial activity of *A. indica* ethanolic leaf extract against Gram-positive and Gram-negative bacteria

Name of Microorganism		Diameter of Zone of Inhibition (mm)								
		0 mg	1 mg	2 mg	3 mg	4 mg	5 mg	6 mg	7 mg	10 mg
Gram-Positive	*Staphylococcus aureus*	–	–	–	–	8	8	8	10	11
	Streptococcus faecalis	–	–	8	8	11	11	12	12	14
Gram-Negative	*Coliform spp.*	–	–	–	–	–	–	–	8	8
	Escherichia coli	–	–	–	–	–	8	8	8	8
	Klebsiella pneumoniae	–	–	10	10	12	12	14	15	17
	Pseudomonas aeruginosa	–	–	–	–	–	8	8	8	8

*Diameter below 7 mm is omitted, as diameter of well is 7 mm. 0 mg is solvent as negative control.

are given in Table 6. The zones of inhibition produced by these extracts were ranged from 8-17 mm for different concentrations for different strains. Maximum zone of inhibition produced by leaf extract was 17 mm at 10 mg/ml concentration.

DISCUSSION

Infection-causing-bacteria are rapidly becoming resistant to conventional drugs for example Methicillin-and Vancomycin-resistant *Staphylococcus aureus* (MRSA/VRSA).[25] As the global scenario is now changing towards the use of nontoxic plant products having traditional medicinal use, development of modem drugs from *A. indica* should be emphasized for the control of various diseases. In fact, time has come to make good use of centuries-old knowledge on *A. indica (neem)* through modern approaches of drug development. Previously it has been observed that, oil from the leaves, seeds and bark possesses a wide spectrum of antibacterial action against Gram-negative and Gram-positive microorganisms, including *M. tuberculosis* and streptomycin resistant strains [26]. In this study, ethanolic extract of *A. indica* leaf extract was investigated for its potential bioactivity and antibacterial activity. To ascertain any bioactivity of desired plant, it should be affirmed that whether this plant has bioactive compound or not. Brine shrimp lethality assay considered a useful tool for preliminary assessment of toxicity and Bioactivity of a given plant extracts. From the assay, LC_{50} value of the extract was determined and it was 37.15 µg/ml. The determined LC_{50} value of this extract was found to be quite lower than the previous studies[26] indicating that the prepared extract was rich in bioactive compounds. The ethanolic extract of neem leaf has been shown to cause cell death of prostate cancer cells (PC-3) by inducing apoptosis as evidenced by a dose-dependent increase in DNA fragmentation and a decrease in cell viability.[27] Nimbolide , a limonoid isolated from Azadirachta indica, is the chief cytotoxic principle in Neem leaf extract being used as a lead compound for anti-cancer analogue design.[28] This clearly indicates the presence of potent bioactives which might be very useful as antiproliferative, antitumor, pesticidal and other bioactive agents.

Then the ethanolic leaf extract of *A. indica* was tested against selected Gram-positive and Gram-negative bacteria. To assure whether the collected strains were antibiotic resistant or not, first of all, antibiogram of these strains was determined and found that almost all strains showed resistance or immediately sensitive against all the standard antibiotics used. However the antibacterial effect of the leaf extract was organism and concentration dependent. Ethanolic extract of leaf was more active towards the Gram positive strain and less active towards the Gram negative strain used in present studies (Table 6). Among the different bacteria tested maximum inhibition was found in *Klebsiella pneumoniae* followed by *Streptococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*. However, *Coliform spp.* remained less sensitive at low concentration but become sensitive at higher concentration (7mg/ml). The striking and distinctive feature of observed antibacterial activity of *A. indica* extract is that it exhibited strong activity against multi-drug resistant bacteria *Klebsiella pneumoniae* at very low concentration (2mg/ml). From the previous study, it was observed that, ethanol extract showed good inhibitory activity with low MIC concentration as compared to other extracts. Ethanol has higher polarity when compared with other solvents and hence the antibacterial compounds are polar compounds which are under purification process. Ethanolic extract of neem leaf shows significant antibacterial activity against *Staphylococcus aureus, E. coli, Candida albicans, Aspergillus niger and Penicilium citrinum.*[29] Another study showed that the extract of the leaf of *Azadirachta indica* exhibited pronounced activity against *Bacillus subtilis*, high activity against the Gram-positive *Staphylococcus aureus,* and low activity against *Escherichia coli.*[30] Previous studies showed similar result with this one incase of *Staphylococcus aureus* and *Escherichia coli.*

CONCLUSION

The findings of this study indicate that the leaf extract of *Azadirachta indica* has potential activities to bacteria. Although crude extracts from various parts of *A. indica* (neem) have medicinal applications from time immemorial, modern drugs can be developed after extensive investigation of its

bioactivity, mechanism of action, pharmacotherapeutics, and toxicity after proper standardization and clinical trials. An extensive research is required on *A. indica* and its product for therapeutic utilization.

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Evaluation of the antibacterial activity and toxicity of *Terminalia ferdinandia* fruit extracts

I. E. Cock[a,b]*, S. Mohanty[a]

[a]*Biomolecular and Physical Sciences, Nathan Campus, Griffith University, 170 Kessels Rd, Nathan, Brisbane, Queensland 4111, Australia.*
[b]*Genomics Research Centre, Gold Coast Campus, Griffith University, Parklands Drive, Southport, Queensland 4222, Australia*

ABSTRACT

Introduction: *Terminalia ferdinandiana* is an endemic Australian native plant with a history of use as a food and as a medicinal agent by indigenous Australians. Yet the medicinal bioactivities of this plant are poorly studied. In the current study, solvent extracts from *T. ferdinandiana* fruit pulp were tested for antimicrobial activity and toxicity *in vitro*. **Results:** All extracts displayed antibacterial activity in the disc diffusion assay. The methanol extract proved to have the broadest specificity, inhibiting the growth of 13 of the 14 bacteria tested (92.9%). The deionised water extract inhibited the growth of 11 of the 14 bacteria tested (78.6%). The ethyl acetate, chloroform and hexane extracts inhibited 21.4%, 28.6% and 14.3% respectively. *T. ferdinandiana* methanolic extracts were approximately equally effective against Gram-positive (100%) and Gram-negative bacteria (90%). All other extracts were more effective at inhibiting the growth of Gram-positive bacteria. The water, ethyl acetate, chloroform and hexane extracts inhibited the growth of 100, 50, 50 and 50% Gram-positive bacteria respectively. In contrast, they inhibited the growth of 70, 10, 20 and 0% Gram-negative respectively. All *T. ferdinandiana* extracts were either non-toxic (ethyl acetate, chloroform, hexane) with no significant increase in mortality induction, or of low toxicity (LC_{50} >1000 µg/ml) (methanol, deionised water) in the *Artemia fransiscana* bioassay. **Conclusions:** The low toxicity of the *T. ferdinandiana* extracts and their inhibitory bioactivity against bacteria validate Australian Aboriginal usage of *T. ferdinandiana* and indicates its medicinal potential as well as its potential as a source of natural ascorbic acid.

Key words: *Terminalia ferdinandiana*, Kakadu plum, antibacterial, medicinal plants, phytotoxicity, superfoods

INTRODUCTION

Terminalia ferdinandiana (commonly known as Kakadu plum, gubinge, bush plum, billy goat plum and salty plum) is a moderately sized semi-deciduous tree of the family Combretaceae.[1] It is endemic to Australia, occurring predominantly in the tropical grasslands of the Northern Territory and the Kimberley region of Western Australia. *T. ferdinandiana* flowers at the end of dry season (September-November) and develops fruits from the middle of the wet season (January-June) to the early part of dry season. The fruit are 1.5 to 2 cm long ovoid shaped smooth fleshy drupes with a short beak at the tip. They become yellow to green in colour when ripe. The fruit have been used as a food source by Australian Aborigines in the northern regions of Australia for thousands of years.[2-4] They are astringent and have a pleasant but tart, slightly bitter flavour when eaten fresh[5] and are increasingly being used to produce powders, sauces, jams, beverages and preserves, as well as in cosmetic products.

T. ferdinandiana also has a history of use as a traditional medicine for the treatment of numerous ailments. The fruit were eaten by Australian Aborigines on long treks or hunting trips and was considered more valuable as a medicine rather than as a food.[6-8] The inner bark of the tree was also used medicinally to treat a variety of skin disorders and infections including wounds, sores and boils.[2] It is also effective in controlling fungal infections such as ringworm, and in the treatment of bacterial infections including its use in treating leprosy.[2]

Recently, *T. ferdinandiana* has been attracting attention due to its interesting phytochemistry. In particular, extremely high levels of ascorbic acid (vitamin C) have been reported for *T. ferdinandiana* fruit.[9,10] Indeed, *T. ferdinandiana* is now known as the richest source of vitamin C of any fruit in the world, with levels over 900 times higher than the same weight of blueberries.[10] Some studies have estimated the levels of

Address for correspondence:
Tel.: +61 7 37357637; fax: +61 7 37355282.
E-mail: I.Cock@griffith.edu.au (I. E. Cock).

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ascorbic acid in *T. ferdinandiana* fruit to be as high as 5.5% of dry weight, in comparison to approximately 0.5% dry weight in oranges, grapefruit and limes.[3] Ascorbic acid is well known for its ability to scavenge free radicals and thereby reduce oxidative stress.[11] As the induction of oxidative stress is known to be associated with some cancers, cardiovascular disease, neurodegeneration disorders, diabetes and obesity,[12] the high levels of ascorbic acid associated with *T. ferdinandiana* fruit may also have beneficial health related bioactivities.

Phytochemical studies of the nutritional value of *T. ferdinandia* fruit have also shown it to also be high in other important polyphenolic antioxidants including ellagic and gallic acids.[13] Pure ellagic and gallic acids and their derivatives have previously been shown to have antibacterial,[14,15] antifungal,[16,17] antiviral,[18] anti-inflammatory,[19] antimutagenic,[20] and antiallergic bioactivities.[21] Furthermore, ellagic and gallic acids have demonstrated cytotoxic activity towards cancer cells, whilst being nontoxic to normal cell lines.[22-23]

Given the previous phytochemical studies, it is surprising that the therapeutic potential of *T. ferdinandiana* remains largely unstudied. Most of the studies regarding this plant solely report on the vitamin C level and the total antioxidant capacity without examining medicinally important bioactivities. Therefore, the current study reports on the antibacterial properties of *T. ferdinandiana* fruit pulp extracts as well as examining their toxicity to determine their potential as antibiotic agents and to validate the ethnopharmacological usage by Australian Aborigines from northern regions of Australia.

MATERIALS AND METHODS

Plant material

T. ferdinandiana *fruit pulp samples*

T. ferdinandiana fruit pulp was a gift from David Boehme of Wild Harvest, Northern Territory, Australia. The pulp was frozen for transport and stored at –10°C until processed.

Preparation of crude extracts

T. ferdinandiana fruit pulp was thawed at room temperature and dried in a Sunbeam food dehydrator. The dried pulp material was subsequently ground to a coarse powder. 1 g of each of the ground dried pulp was extracted extensively in 50 ml of either methanol, deionised water, ethyl acetate, chloroform or hexane for 24 hours at 4°C with gentle shaking. All solvents were supplied by Ajax and were AR grade. The extracts were filtered through filter paper (Whatman No. 54) under vacuum followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant pellets were dissolved in 10 ml deionised water. The extract was passed through 0.22 μm filter (Sarstedt) and stored at 4°C.

Antibacterial screening

Test microorganisms

All microbial strains were obtained from Michelle Mendell and Tarita Morais, Griffith University, Australia. Stock cultures of *Aeromonas hydrophilia*, *Alcaligenes feacalis*, *Bacillus cereus*, *Citrobacter freundii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Salmonella newport*, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* were subcultured and maintained in nutrient broth at 4°C.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified Kirby-Bauer disc diffusion method.[24] Briefly, 100 μl of the test bacteria were grown in 10 ml of fresh media until they reached a count of approximately 10^8 cells/ml. 100 μl of microbial suspension was spread onto nutrient agar plates.

The extracts were tested using 5 mm sterilised filter paper discs. Discs were impregnated with 10 μl of the test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4°C for 2 hours before incubation with the test microbial agents. Plates inoculated with *Alcaligenes feacalis*, *Aeromonas hydrophilia*, *Bacillus cereus*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Proteus mirabilis, Pseudomonas fluorescens* and *Serratia marcescens* were incubated at 30°C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *Escherichia coli*, *Salmonella newport*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* were incubated at 37°C for 24 hours, then the diameters of the inhibition zones were measured. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate. Mean values are reported in this study. Standard discs of ampicillin (2 μg) and chloramphenicol (10 μg) were obtained from Oxoid Ltd. and served as positive controls for antimicrobial activity. Filter discs impregnated with 10 μl of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the *T. ferdinandiana* extracts was determined by the disc diffusion method across a range of doses. The plant extracts were diluted in deionised water across a concentration range of 5 mg/ml to 0.1 mg/ml. Discs were impregnated with 10 μl of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the MIC values.

Toxicity Screening

Reference toxins for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 1.6 mg/ml solution in distilled water and was serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay. Mevinphos (2-methoxycarbonyl-1-methylvinyl dimethyl phosphate) was obtained from Sigma-Aldrich as a mixture of cis (76.6%) and trans (23.0%) isomers and prepared as a 4 mg/ml stock in distilled water. The stock was serially diluted in artificial seawater for use in the bioassay.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using the *Artemia franciscana* nauplii lethality assay developed by Meyer et al[25] for the screening of active plant constituents with the following modifications. *Artemia franciscana* Kellogg cysts were obtained from North American Brine Shrimp, LLC, USA (harvested from the Great Salt Lake, Utah). Synthetic seawater was prepared using Reef Salt, AZOO Co., USA. Seawater solutions at 34 g/l distilled water were prepared prior to use. 2 g of *A. franciscana* cysts were incubated in 1 l synthetic seawater under artificial light at 25°C, 2000 Lux with continuous aeration. Hatching commenced within 16-18 h of incubation. Newly hatched *A. franciscana* (nauplii) were used within 10 h of hatching. Nauplii were separated from the shells and remaining cysts and were concentrated to a suitable density by placing an artificial light at one end of their incubation vessel and the nauplii rich water closest to the light was removed for biological assays. 400 µl of seawater containing approximately 42 (mean 41.6, $n = 150$, SD 17.8) nauplii were added to wells of a 48 well plate and immediately used for bioassay. The plant extracts were diluted to 2 mg/ml in seawater for toxicity testing, resulting in a 1 mg/ml concentration in the bioassay. 400 µl of diluted plant extracts and the reference toxins were transferred to the wells and incubated at 25 ± 1°C under artificial light (1000 Lux). A negative control (400 µl seawater) was run in at least triplicate for each plate. All treatments were performed in at least triplicate. The wells were checked at regular intervals and the number of dead counted. The nauplii were considered dead if no movement of the appendages was observed within 10 seconds. After 72 h all nauplii were sacrificed and counted to determine the total number per well. The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.[26]

RESULTS

Antibacterial Activity

1 kg of *T. ferdinandiana* fruit pulp was dehydrated resulting in 165 g of dried material. Extraction of 1 g of dried plant material with various solvents yielded dried plant extracts ranging from 23 mg to 498 mg (Table 1). Methanol and deionised water both gave high yields of dried extracted material (371 and 498 mg respectively) whilst ethyl acetate, chloroform and hexane all extracted relatively low masses (28, 60 and 23 mg respectively). The dried extracts were resuspended in 10 ml of deionised water resulting in the extract concentrations shown in Table 1.

10 µl of each extract (50 µg) was tested in the disc diffusion assay against 14 bacteria (Table 2). *T. ferdinandiana* fruit methanol extract was particularly effective as an antibacterial agent, inhibiting the growth of 13 of the 14 bacteria tested (92.9%). The deionised water extract also displayed broad antibacterial activity, inhibiting the growth of 11 of the 14 bacteria tested (78.6%). Ethyl acetate, chloroform and hexane extracts each had narrower specificity, inhibiting 3 (21.4%), 4 (28.6%) and 2 (14.3%) of the tested bacteria respectively.

Both Gram-positive and Gram-negative bacteria were affected approximately equally by the *T. ferdinandiana* pulp methanol extract (90% and 100% respectively). In contrast, all other extracts proved more effective at inhibiting the growth of Gram-positive bacteria. Of the 10 Gram-negative bacteria tested, 7 (70%) were inhibited by the *T. ferdinandiana* pulp deionised water extract whilst 100% of the Gram-positive bacterial growth was inhibited by this extract. The antibacterial specificity towards Gram-positive bacteria was even more evident for the ethyl acetate, chloroform and hexane extracts. The ethyl acetate extract inhibited the growth of 2 of the 4 Gram-positive bacteria tested (50%) and 1 of the 10 Gram-negative bacteria tested (10%). The chloroform and hexane extracts also inhibited the growth of 2 of the 4 Gram-positive bacteria tested (50%) each. In contrast, the chloroform extract inhibited the growth of 2 of the 10 Gram-negative bacteria tested (20%), whilst no Gram-negative bacterial growth was inhibited by the hexane extract (0%).

The relative level of antibacterial activity was evaluated by determining the MIC values for each extract against the bacteria which were shown to be susceptible by disc

Table 1: The mass of dried material extracted with the various solvents and the concentration after resuspension in deionised water

Solvent	Mass of Dried Extract (mg)	Resuspended Extract Concentration (mg/ml)
Methanol	371	37.1
Deionised Water	498	49.8
Ethyl Acetate	28	2.8
Chloroform	60	6
Hexane	23	2.3

diffusion assays. MIC's were evaluated in the current studies by disc diffusion across a range of concentrations. This has previously been determined to be a valid method of MIC determination as MIC values determined by disc diffusion correlate well with those determined by broth dilution assays.[27]

The methanol extract was particularly effective at inhibiting the growth of *A. faecalis*, *P. mirabilis*, *P. fluorescens*, *S. newport* and *S. pyogenes*, as seen by minimum inhibitory concentration (Table 3). Indeed, the growth of these bacteria was inhibited by low concentrations (<100 µg/ml) of the extract. *A. hydrophilia*, *C. freundi*, *S. sonnei*, *B. cereus*, *S. aureus* and *S. epidermidis* also were quite susceptible, displaying inhibited growth at concentrations below 500 µg/ml. *P. mirabilis* growth was also inhibited by low concentrations (<100 µg/ml) of the deionised water extract, whilst *C. freundi*, *E. coli*, *P. fluorescens* and *S. epidermidis* were all inhibited by the water extract at concentrations below 500 µg/ml.

Qantification of toxicity

The *T. ferdinandiana* fruit extracts (Figures 1a-e) were diluted in artificial seawater for toxicity testing in the *Artemia franciscana* lethality bioassay. For comparison, the reference

Table 2: Antibacterial activity of *T. ferdinandiana* fruit extracts measured as zones of inhibition (mm)

	Methanol extract	Water extract	Ethyl acetate extract	Chloroform extract	Hexane extract	Ampicillin	Chloramphenicol	Negative control (water)
Gram negative rods								
A. faecalis	13.0 ± 0	–	–	–	–	15.2 ± 1.2	6.3 ± 0.6	–
A. hydrophilia	8.0 ± 0	7.3 ± 1.2	–	6.0 ± 0	–	12.0 ± 1.0	28.7 ± 1.6	–
C. freundi	12.7 ± 1.2	13.6 ± 1.2	–	–	–	8.3 ± 0.6	15.7 ± 1.2	–
E. coli	8.3 ± 0.6	7.8 ± 1.0	–	–	–	14.7 ± 0.6	17.3 ± 0.6	–
K.pneumoniae	6.0 ± 0	–	–	–	–	10.3 ± 0.6	21.3 ± 1.5	–
P. mirabilis	14.7 ± 1.5	12.3 ± 0.6	7.0 ± 1.0	7.7 ± 0.6	–	17.3 ± 0.6	8.7 ± 0.6	–
P. fluorescens	12.7 ± 0.6	9.7 ± 0.6	–	–	–	18.2 ± 0.5	21.2 ± 1.2	–
S. newport	12.7 ± 0.6	7.0 ± 0	–	–	–	18.7 ± 0.6	20.3 ± 0.6	–
S. marcescens	–	–	–	–	–	0 ± 0	14.7 ± 0.6	–
S. sonnei	9.7 ± 0.6	7.3 ± 0.6	–	–	–	14.0 ± 0	14.3 ± 0.6	–
Gram positive rods								
B. cereus	11.7 ± 0.6	7.3 ± 0.6	–	–	–	26.7 ± 0.6	13.3 ± 1.2	–
Gram positive cocci								
S. aureus	8.3 ± 0.6	6.6 ± 0.6	6.0 ± 0	7.2 ± 1.0	6.7 ± 0.6	11.7 ± 2.1	16.0 ± 1.0	–
S. epidermidis	10.7 ± 0.6	14.3 ± 0.6	9.0 ± 0	6.0 ± 0	6.3 ± 0.6	26.3 ± 1.5	12.3 ± 0.6	–
S. pyogenes	12.7 ± 1.2	7.3 ± 0.6	–	–	–	17.0 ± 1.0	24.0 ± 1.0	–

Numbers indicate the mean diameters (mm) of inhibition of triplicate experiments ± standard deviation. – indicates no growth inhibition. Chloramphenicol (10 µg) and ampicillin (2 µg) were used as the positive controls.

Table 3: Minimum inhibitory concentrations (µg/ml) of *T. ferdinandiana* extracts against susceptible bacteria

	Methanol	Water	Ethyl Acetate	Chloroform	Hexane
A. faecalis	46.8	–	–	–	–
A. hydrophilia	160.2	518.8	–	695.5	–
C. freundi	159.4	287.1	–	–	–
E. coli	684.3	348.8	–	–	–
K. pneumoniae	924.7	–	–	–	–
P. mirabilis	29.1	85.9	500	672.3	–
P. fluorescens	47.3	147.1	–	–	–
S. newport	35	875.7	–	–	–
S. sonnei	112.5	566.6	–	–	–
B. cereus	113.5	530.9	–	–	–
S. aureus	285.6	756.8	825.7	707.1	594.6
S. epidermidis	114.7	196.7	739.7	695.6	347.9
S. pyogenes	47.6	250	–	–	–

Numbers indicate the mean MIC values of at least triplicate determinations. – indicates no growth inhibition.

toxins potassium dichromate (800 µg/ml) (Figure 1g) and Mevinphos (2000 µg/ml) (Figure 1h) were also tested in the *Artemia franciscana* lethality bioassay. The potassium dichromate and Mevinphos reference toxins were much more rapid in their onset of mortality than any of the *T. ferdinandiana* extracts at the concentrations tested. For both reference toxins, the induction of mortality was seen within the first 3 hours of exposure. 100% mortality was

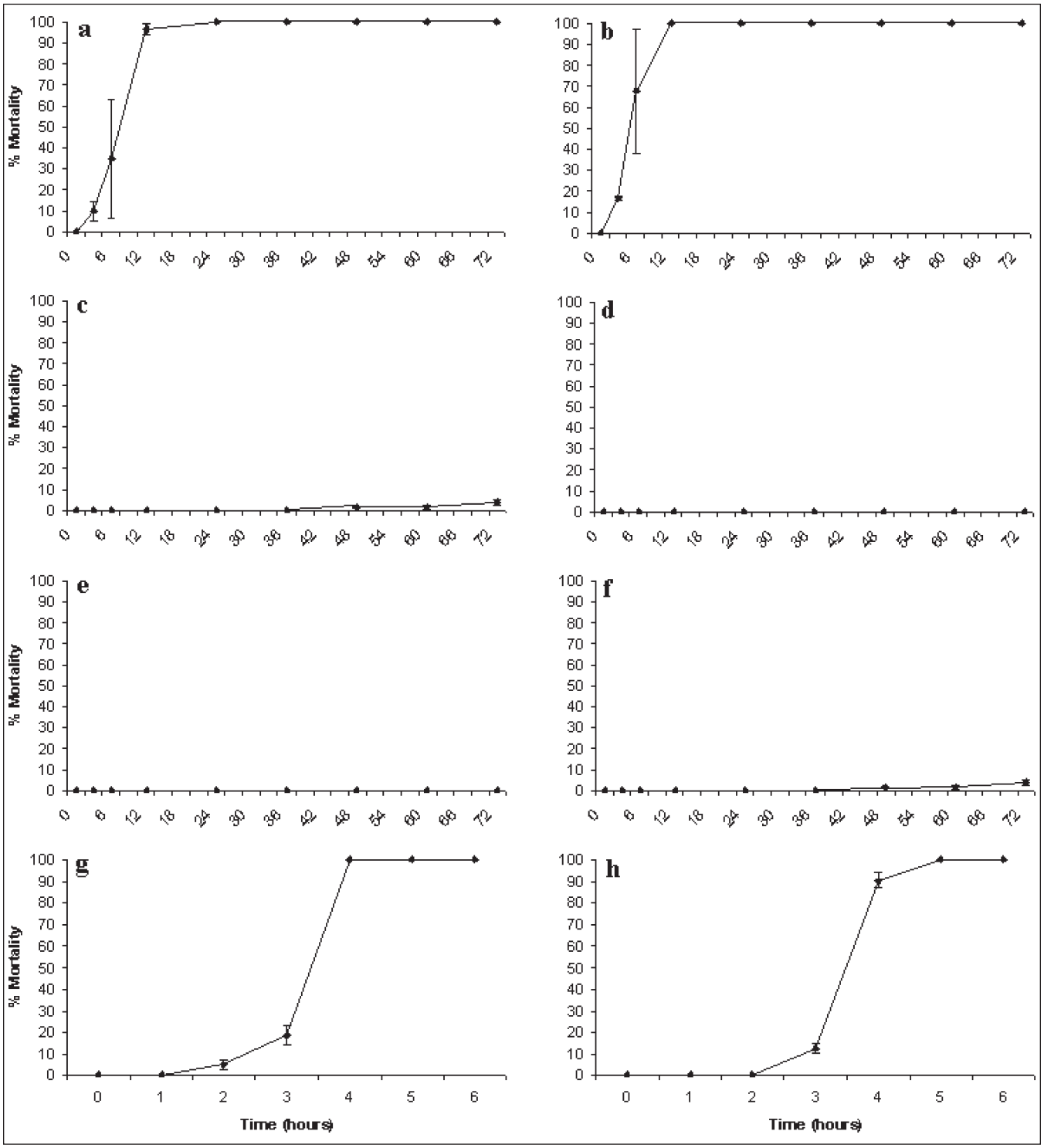

Figure 1: Brine shrimp lethality of (a) *T. ferdinandiana* fruit methanol extract (1000 µg/ml), (b) *T. ferdinandiana* fruit water extract (1000 µg/ml), (c) *T. ferdinandiana* fruit ethyl acetate extract (1000 µg/ml), (d) *T. ferdinandiana* fruit chloroform extract (1000 µg/ml), (e) *T. ferdinandiana* fruit hexane extract (1000 µg/ml), (f) artificial seawater negative control, (g) potassium dichromate (800 µg/ml), (h) Mevinphos (2000 µg/ml). All bioassays were performed in at least triplicate and are expressed as mean ± standard deviation.

Table 4: LC_{50} (95% confidence interval) for *A. franciscana* nauplii exposed to *T. ferdinandiana* leaf and fruit extracts, the reference toxins potassium dichromate and Mevinphos and a seawater control

	LC50				
	3 hours	6 hours	24 hours	48 hours	72 hours
Methanol	1964	1473	913	900	886
Water	1963	1050	900	900	900
Ethyl Acetate	–	–	–	–	–
Chloroform	–	–	–	–	–
Hexane	–	–	–	–	–
Potassium Dichromate	–	286	92	86	83
Mevinphos	–	1286	1004	525	109
Seawater Control	–	–	–	–	–

– denotes values that were not obtained as ≥ 50% mortality was not obtained at this time point.

evident following 4 hours of exposure. In contrast, mortality due to *T. ferdinandiana* methanol and water extract exposure was evident within 6 hours and 12 hours was required to achieve approximately 100% mortality. None of the other extracts induced mortality above the levels seen for seawater controls at any time tested.

To determine the effect of toxin concentration on the induction of mortality, the LC_{50} values of the extracts was determined by testing across the concentration range 2000 µg/ml to 15 µg/ml in the *Artemia* nauplii bioassay. For comparison, potassium dichromate and Mevinphos were tested across the same concentration range. Table 4 shows the LC_{50} values of *T. ferdinandiana* extracts towards *A. franciscana*. No LC_{50} values are reported for the *T. ferdinandiana* ethyl acetate, chloroform or hexane fruit extracts as no increase in mortality above the seawater controls was seen for these extracts at any time tested. The *T. ferdinandiana* methanol and water extracts displayed similar toxicity to Mevinphos at 24 hours but were substantially less toxic at 48 and 72 hours with 48 hour LC_{50} values of 900 µg/ml for both the methanol and water extracts and 72 hour LC_{50} values of 886 µg/ml and 900 µg/ml respectively, compared to 48 h and 72 h LC_{50} values of 525 µg/ml and 109 µg/ml for Mevinphos. Potassium dichromate was substantially more toxic at 24 hours (24 h LC_{50} 92 µg/ml), 48 hours (48 h LC_{50} 86 µg/ml) and 72 hours (72 h LC_{50} 83 µg/ml).

DISCUSSION

The current study reports on the antimicrobial activity and toxicity of *T. ferdinandiana* fruit pulp extracts. The ability of *T. ferdinandiana* fruit pulp extracts to inhibit the growth of both Gram-positive and Gram-negative bacteria seen in this study is in agreement with previous reports of the antibacterial activity of other plants used by Australian Aborigines as antibacterial agents. The antiseptic properties of *Eucalypts*,[28-31] *Leptospermums*,[32-35] and *Melaleucas*,[36,37] have been extensively studied and shown to inhibit the growth of a wide variety of both Gram-positive and Gram-negative bacteria.

We report *T. ferdinandiana* fruit pulp solvent extracts to have greater antibacterial activity towards Gram-positive bacteria than towards Gram-negative bacteria in this study. The greater susceptibility of Gram-positive bacteria towards the *T. ferdinandiana* fruit extracts is in agreement with previously reported results for South American,[38] African,[39-40] and Australian[41] plant extracts. Results within this laboratory have also confirmed the greater susceptibility of Gram-positive bacteria towards other Australian plant extracts.[28] The Gram-negative bacterial cell wall outer membrane is thought to act as a barrier to many substances including antibiotics.[42] The uptake of the *T. ferdinandiana* extract antibiotic agents by Gram-negative bacteria is presumably affected by the cell wall outer membrane of some bacteria.

Individual *T. ferdinandiana* fruit pulp components responsible for the extracts antiseptic potential were not identified in the current study. However, previous reports have demonstrated that *T. ferdinandiana* contain high levels of antioxidants.[9,10,13] In particular, several studies have highlighted the fact the *T. ferdinandiana* has the highest recorded concentrations of ascorbic acid of any fruit in the world.[9,10] However, it is unlikely that ascorbic acid alone is responsible for the broad antibacterial activity and low MIC values seen during this study, even at the high levels present in *T. ferdinandiana* fruit. Previous studies have demonstrated that ascorbic acid alone displays only weak antibacterial activity towards *E. coli* and *S. aureus*, even at relatively high concentrations.[43] Instead, if ascorbic acid is involved in the antibacterial bioactivities reported here, it is more likely that it works in a synergistic manner with other *T. ferdinandiana* extract phytochemicals. Ascorbic acid has previously been shown to enhance the antibacterial activity of other polyphenolic compounds through an inhibition of the oxidation of these polyphenols. For

example, epigallocatechin gallate (EGCG), the most abundant polyphenol of tea leaves (*Camellia sinensis*) has well established inhibitory activity towards *S. aureus* growth although this activity is unstable due to oxidation.[44,45] The addition of ascorbic acid to EGCG solutions has been shown to significantly enhance their antibacterial activity and to prolong their inhibitory effect.[46]

Gallic and ellagic acids, as well as their derivatives, have been reported to be present in *T. ferdinandiana* fruit.[13] As these compounds have well established antibacterial activities,[14,15] they may be responsible, at least in part, for the bacterial growth inhibitory effects of *T. ferdinandiana* fruit reported here. Similarly, gallic and ellagic acids also have well documented antifungal,[16,17] antiviral,[18] anti-inflammatory,[19] antimutagenic,[20] antiallergic[21] and anticancer[22,23] bioactivities. Further studies to examine *T. ferdinandiana* fruit extracts against these bioactivities is also warranted.

T. ferdinandiana fruit has also been reported to contain a number of other important phytochemical components, vitamins and nutrients which could contribute to medicinally important bioactivities of this plant, including antibacterial activity. Whilst *T. ferdinandiana* fruit extracts are not yet fully characterised due to difficulties in separating some components, high levels of antioxidant molecules have been reported. Apart from the high ascorbic, gallic and ellagic acid levels previously discussed, *T. ferdinandiana* fruit also contains high levels of phenolic compounds. Indeed, phenolic compound levels nearly 5 fold higher than in blueberries have previously been demonstrated to be associated with polar *T. ferdinandiana* fruit extracts.[9] These authors noted *T. ferdinandiana* fruit to be very rich in chlorophyll a and also to have high levels of chlorophyll b. Both chlorophyll a and b have previously been shown to be capable of relieving oxidative stress.[47] Lipophilic *T. ferdinandiana* fruit extracts are rich in lutein (a carotenoid antioxidant compound associated with eye health) and with vitamin E and vitamin E analogues.[9] Other antioxidants present in *T. ferdinandiana* fruit include the glucosides quercetin and hesperitin, and the glycosides kaempferol and luteolin.[9] *T. ferdinandiana* fruit is also a good source of the minerals magnesium, zinc, calcium, potassium, sodium, iron, phosphorous, manganese, copper and molybdenum.[9] Of further interest, the same study also noted a high potassium/sodium ratio in *T. ferdinandiana* fruit.[9] As high potassium/sodium ratios have been shown to relieve hypertension,[48] testing the effect of *T. ferdinandiana* fruit on individuals suffering from this condition is also warranted.

The findings reported here also indicate that *T. ferdinandiana* fruit extracts display low toxicity towards *Artemia franciscana*. Indeed, the ethyl acetate, chloroform and hexane extracts did not induce mortality above that seen for the seawater control at any dose or time tested. Only the methanol and deionised water extracts were seen to induce mortality above that of the seawater controls and even this is considered low toxicity. Both of these extracts displayed 24, 48 and 72 h LC_{50} values of approximately 900 µg/ml. As an LC_{50} of ≥1000 µg/ml is defined as nontoxic,[25] these extracts are considered of only low toxicity. Toxicity towards *A. franciscana* has also previously been shown to correlate well with toxicity towards human cells for some toxins.[49] Therefore, studies into potential anticancer activities of *T. ferdinandiana* fruit extracts are warranted, particularly for the methanol and water extracts.

In conclusion, this study focussed on the bacterial growth inhibitory potential of *T. ferdinandiana* fruit pulp. Other studies are needed to examine other medicinally important bioactivities of *T. ferdinandiana* fruit. The results of the current study indicate that *T. ferdinandiana* fruit pulp extracts are worthy of further study due to their antibacterial activity. Evaluation of *T. ferdinandiana* fruit pulp extract antibacterial properties against a more extensive panel of microbial agents is warranted. Likewise, purification and identification of the bioactive components is needed to examine the mechanisms of action of these agents. Whilst the extracts examined in this report are promising as antimicrobial agents, caution is needed before these compounds can be applied to medicinal purposes and as food additives to inhibit spoilage. In particular, further toxicity studies using human cell lines are needed to determine the suitability of these extracts for these purposes.

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Assessment of the Antimicrobial Potency of Leaf Extracts from *Vitex Nugundo* and *Gloriosa Superba*

K. Choudhary[1]*, N. Mathur[1], A. Chaudhary[2] and B.L Chaudhary[2]

[1]*Department of Botany and Biotechnology, LM College of Science and Technology, Jodhpur-342003, India.*
[2]*Department of Botany, MLS University, Udaipur-313001, India.*

ABSTRACT

An increase in the number of antibiotic resistant strains makes the discovery of new therapeutic agents critically important. During present study antimicrobial effects of the leaf extracts of *Vitex nugundo* (VN) and *Gloriosa superba* (GS) in combination with chloramphenicol, on *Escherichia coli, Salmonella typhi, Staphlococcus aureus* and *Candida albicans* was investigated. For this purpose, the dilution sensitivity and disc diffusion techniques were respectively applied in determining minimum inhibitory concentration (MIC) of the plant extracts, and the sensitivities of the organisms to the plant extracts and their combinations with chloramphenicol. *V. nugundo* and *G. superba* showed very high antimicrobial activity against all the test organisms. In combination, the effect of VN on *E. coli* and *S. typhi* was completely antagonized by that of GS whereas additive effect on *S. aureus* and *C. albicans* was observed, indicating that the combination of VN and GS might be effective against gram positive pathogenic organisms. The combination of either plant extract with chloramphenicol produced synergistic effect on only *C. albicans*. The smaller MIC of GS indicated greater effectivity than VN. It is concluded that the additive effect produced by the combination of the two plant extracts, and the synergic effect from the combination of any of the extracts with chloramphenicol, offer alternative therapy to gram positive bacterial infections and candidiasis respectively.

Key Words: Antibacterial, *Candida albicans*, Chloramphenicol, Microorganisms.

INTRODUCTION

Antibiotics provide the main basis for the therapy of microbial (bacterial and fungal) infections. Since the discovery of these antibiotics and their uses as chemotherapeutic agents there was a belief in the medical fraternity that this would lead to the eventual eradication of infectious diseases. However, overuse of antibiotics has become the major factor for the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms. An increase in the number of antibiotic resistant strains makes the discovery of new therapeutic agents critically important. Many of the currently used anti-infective and antineoplastic agents are natural products, initially isolated from plants[1,2] and algae.[3,4] Recently there is an increasing concern and the need to source for locally available drugs because of un-affordability of conventional chemotherapeutic agents and clinical cost. Apparently this situation has generated a few studies on the phytochemistry and the medicinal potency of some of the medicinal plants known to aborigins. Besides, the current wave of antimicrobial resistance to chemotherapeutic drugs is of global concern.[5] There is need, therefore, to search for such plants that could be resistance-free.

Vitex negundo (VN) Linn (verbenaceae), a large aromatic shrub with typical five foliate leave pattern, is found throughout the greater part of India at warmer zones and ascending to an altitude of 1500 m in outer, Western Himalayas. It has been claimed to possess many medicinal properties.[6] Leaves of VN have been investigated for its anti-inflammatory activity in past,[8-12] including its mechanism of action.[7,11] Similarly, fresh leaves of VN have been suggested to possess antiinflammatory and pain suppressing activities mediated via PG synthesis inhibition, antihistamine, membrane stabilization and antioxidant activities.[11] VN has also been reported to possess anti-ulcer activity against piroxicam induced ulcers, probably by increasing PG levels.[12]

Gloriosa superba (GS) Linn. (liliaceae), is one of the endangered species among the medicinal plants[13]

Address for correspondence:
Ph.: +91-9460660533
E-mail: kchoudharylmc@gmail.com

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which is a striking tuberous climbing plant with brilliant wavy edged yellow and red flowers that appears from November to March every year.[14] *G. superba* is a native of tropical Africa and is now found growing naturally in many countries of tropical Asia including Bangladesh, India, Sri Lanka, Malaysia and Myanmar. It is one of the seven upavishas (semi-poisonous drugs) in the Indian medicine, which cure many ailments but may prove fatal on misuse.[15] The tuberous root stocks of glory lily, *G. superba* boiled with *Sesamum* oil is applied twice a day on the joints, affected with arthritis reduces pain.[16] It is also used to treat intestinal worms, bruises, infertility, skin problem and impotence. The sap from the leaf tip is used as a smoothening agent for pimples and skin eruptions. The tuberous roots are useful in curing inflammation, ulcers, scrofula, bleeding piles, white discharge, skin diseases, leprosy, indigestion, helminthiasis, snake bites, baldness, intermittent fever and debility. It is also considered useful in promoting labor and expulsion of placenta. Seeds are used for relieving rheumatic pain and as a muscle relaxant.[17] The toxins of *G. superba* have an inhibitory action on cellular division resulting in diarrhoea, depressant action on the bone marrow and alopecia. Usually all parts of the plant, especially the tubers are extremely poisonous [18,19] and causes vomiting, purging, stomach ache and burning sensation.[20] The glory lily has been used for suicidal purposes in India, Burma and Eastern Africa due to presence of colchicines.[21,22] The tubers contain colchicines, benzoic and salicylic acid, sterols and resinous substances-colchicines, 3-demethyl colchicine, 1,2-didemethyl colchicine, 2,3-didemethyl colchicine, N-formyl, N-deacetyl colchicines, colchicocide, gloriosine, tannins and superbine.[23] In the world market they are considered as rich sources of colchicines and gloriosine.[24,25]

In carrying out this study, the aim was to evaluate in vitro the antimicrobial potency of *V. nugundo* and *G. superba* by investigating the sensitivities of known pathogenic bacteria, *Escherichia coli, Salmonella typhi, Staphylococcus aureus* and *Candida albicans,* to the plant leaf extracts, individually and in combination with themselves and with a known antibiotic.

MATERIALS AND METHODS

Sources of test organisms and plants

Known cultures of *E. coli, S. typhi, S. aureus* and *C. albicans* were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The cultures were preserved in agar slants until they were used. The test plants were collected from Udaipur District of Rajasthan. They were carried to the herbarium in the Botanical Survey of India, Jodhpur, for identification as *V. nugundo* Linn. and *G. superba* Linn.

Preparation of the plants extracts

After the leaves of the plants were air-dried and grounded in a mortar,[26] the crude extracts of the leaves were prepared using standard procedures.[27,28] This involved soaking 50 g of the powdered extract in 95% ethanol for 48 h at room temperature to allow for maximum extraction of the components. This was followed by evaporation of the filtrate using a rotary evaporator. The residue was retained as the crude extract for each of the test plants and stored in reagent bottles and maintained in the freezer until it was used.

Preparation of extract concentrations for the determination of zones of inhibition

The crude extract (10 mg) was dissolved in 1 mL of dimethyl sulfoxide (DMSO) to obtain a concentration of 10 mg/mL. When 0.1 mL of this solution was dissolved in 9.9 mL DMSO, a solution of concentration 1 mg/mL was obtained. By incorporating 1mL of this final solution into 9 mL of DMSO, a final concentration of 100 µg/mL was obtained. To test the combined extracts, equal volumes (0.1:0.1) were mixed and the mixture was tested along with the individual extracts separately.

Preparation of extract concentrations for minimum inhibitory concentration (MIC) test

The crude extract (100 mg) was dissolved in one mL of DMSO to make a concentration of 100 mg/mL and labeled solution 1.[29] When solution 1 was dissolved in 0.5 mL of DMSO, a concentration of 50 mg/mL was obtained, and this was labeled solution 2. Further, solution 2 was dissolved in 0.5 mL of DMSO to obtain a concentration of 25 mg/mL referred to as solution 3. Solution 4 was obtained by dissolving solution 3 in 0.5 mL of DMSO to give a concentration of 12.5 mg/mL. This process was continued to obtain further concentrations 6.25, 3.12, 1.56, 0.78, 0.39 and 0 mg/mL corresponding to solutions 4 to 9 respectively. By incorporating 1.0 mL of each of solutions 1 to 9 into 9ml Mueller-Hinton broth, final concentrations of 5000, 2500, 1250, 625, 312,156, 78, 39 and 0 mg/mL were obtained for minimum inhibitory concentration (MIC) test.

Preparation of the concentration of a broad-spectrum antibiotic, chloramphenicol, used for test in combination with test plants

The drug used was chloramphenicol 250 mg (Sigma, USA). Chloramphenicol was selected because it is a drug of choice against *S. typhi* and other gram negative enteric bacterial pathogens, and *S. aureus*, a gram positive bacterium.[30] Since *C. albicans* also forms part of the microbial flora of the gastrointestinal tract, it was desirable to test the effect of the combination of chloramphenicol with the local herbs on the bacterial pathogens alongside C. *albicans*. 250 mg of the powdered chloramphenicol was dissolved in deionized water and DMSO as solubilizing agent and

made up to a volume of 25.0 mL at room temperature.[28] This gave a concentration of 10 mg/mL. Further dilutions as with the extracts were made to obtain a solution with a concentration of 1 g/mL. By mixing 1.0 mL of the solution with 9.0 mL of DMSO, a final concentration of 100 µg/mL was obtained. To test the extracts combined with chloramphenicol, equal volumes of extracts and chloramphenicol (0.1:0.1) were mixed and the mixture was tested along with the individual extracts and chloramphenicol separately.

Sensitivity test

To determine the effect of the extracts individually, combined with themselves and with chloramphenicol on the test organisms, a disc diffusion technique using the Kirby-Bauer method was applied in testing pure cultures of the test organisms for their antimicrobial sensitivities based on zones of inhibition on agar plates.[31] In this method, punched circular discs from filter paper (Whatman No.1) that were sterilized in a hot air oven for 1 h were impregnated with 0.1 mL of each of the plant extracts. They were air-dried for a few minutes, and transferred aseptically onto the surface of previously prepared Mueller-Hinton agar plates. This followed incubation at 37°C for 24 h, following which the plates were observed for zones of inhibition.

To test the combined extracts, or extracts combined with chloramphenicol, individual concentrations of the extracts or chloramphenicol were both mixed in equal volumes as earlier described, before impregnating the discs with the combinations. The combinations were tested along with the individual extracts or chloramphenicol separately.

To determine the minimum inhibitory concentration (MIC), a standard inoculum was first prepared by transferring a portion of the pure culture of each isolate into tryptone soya broth (Oxoid CM129) that was incubated at room temperature overnight. The overnight broth culture (0.1 mL) was diluted with 1 mL of distilled water in the ratio of 1:100 to form the standard inoculums[29] following which the dilution susceptibility test technique[30] was applied. This involved inoculating the previously prepared Mueller-Hinton broth containing various concentrations of the extracts of the plants with the standard inoculum.

This was done for each of the test organisms followed by incubation at 37°C for 16 to 20 h. At the end of incubation, the presence or absence of growth for each concentration was recorded. The lowest concentration of the extracts resulting in no growth after 16 to 20 h of incubation was taken as the minimum inhibitory concentration (MIC). The same treatment was given to the combination of the extracts of the two plants and those of the individual extracts and chloramphenicol in the appropriate volume ratio.

Statistical analysis

Differences, if any, in the effectivities of the test plants, singly, in combination with each other and with chloramphenicol, were determined using the statistical method, analysis of variance (ANOVA).[32,33]

RESULTS AND DISCUSSIONS

The effect of the extracts of *V. nugundo* (VN) and *G. superba* (GS) on the test organisms are shown in Table 1. Both VN and GS showed appreciable zones of inhibition (≥15 mm) indicating reasonably good effectivity of each of the plants on test organisms. In the combination of VN and GS, the effect of VN on *E.coli* and *S. typhi* was completely antagonized or masked by that of GS which was not disturbed and remained the same as it was when uncombined. However, the combination of VN and GS produced an enhanced or additive effect on *S. aureus* and *C. albicans*.

The combination of VN and CAF or GS and CAF appeared to produce little additive or enhanced effect on all organisms except *C. albicans* for which there was synergistic effect on the organisms as shown in Table 2. However, there was significant difference ($p < 0.05$) between the plants extracts and their combinations with chloramphenicol, and between the test organisms with respect to their sensitivities to the extracts singly and in combination.

Table 3 shows the minimum inhibitory concentrations (MIC) *V. nugundo* (VN) and *G. superba* (GS), singly and in combination on the test organisms. There was significant difference ($p < 0.05$) between the plants extracts (VN, GS and VN + GS) with respect to minimum inhibitory concentrations (MIC). GS showed advantage over VN (which had higher MIC) whereas there was no significant difference ($p > 0.05$) between GS and VN + GS. There was significant difference ($p < 0.05$) between the test organisms. Both *E. coli* and *S. typhi* were more susceptible to VN and GS than *S. aureus* and *C. albicans*.

Results obtained in this study indicate that *V. nugundo* (VN) and *G. superba* (GS) have very high antimicrobial activity

Table 1: Effect of ethanolic extracts of *V. nugundo* (VN) and *G. superba* (GS) on the test organisms

Test organisms	Zone Size (mm)		
	VN	GS	VN + GS
Escherichia coli	16.6 ± 0.42	20.9 ± 0.43	20.8 ± 0.43
Salmonella typhi	20.3 ± 0.33	25.4 ± 0.35	25.2 ± 0.29
Staphylococcus aureus	15.9 ± 0.28	20.4 ± 0.13	30.5 ± 0.50
Candida albicans	18.2 ± 0.19	21.4 ± 0.15	26.8 ± 0.16

The Figures represents means and standard deviation of triplicate zones of inhibition obtained from 100 µg/mL of extracts.

Table 2: Effect of extracts of *V. nugundo* (VN) and *G. superba* (GS) and chloramphenicol (CAF) on the test organisms

Test organisms	Zone of inhibition (mm)				
	VN	GS	CAF	VN+CAF	GS + CAF
E. coli	16.6 ± 0.42	20.9 ± 0.43	28.3 ± 0.21	31.3 ± 0.27	31.80 ± 0.27
S. typhi	20.3 ± 0.33	25.4 ± 0.35	36.6 ± 0.06	36.6 ± 0.12	39.70 ± 0.18
S. aureus	15.9 ± 0.28	20.4 ± 0.13	21.4 ± 0.04	26.6 ± 0.14	26.60 ± 0.22
C. albicans	18.2 ± 0.19	21.4 ± 0.15	0.00	20.3 ± 0.07	23.50 ± 0.04

The Figures represents means and standard deviation of triplicate zones of inhibition obtained from 100 µg/mL of extracts and chloramphencol.

(zone of inhibition ≥15 mm) against the test organisms, *E. coli, S. typhi, S. aureus and Candida albicans*. Of course, it has been reported that *V. nugundo* (VN) and *G. superba* (GS) have high levels of alkaloids and cardiac glycosides besides anthranoids, anthraquinones, saponins and tannins which are mainly associated with antimicrobial activity.[34,35] The combination of the extracts of the two plants revealed that the effect of *V. nugundo* (VN) on *E. coli* and *S. typhi* was completely antagonized (or masked) by that of *G. superba* (GS), the effect of which remained the same as it was when uncombined. However, the combination produced an enhanced (or additive) effect on *S. aureus* and *C. albicans*. This implies that the combination of the extracts of the two plants may be effective for the treatment of infections from gram positive organisms and may not be effective for the treatment of infections from gram negative organisms.

Little additive effects on all the organisms were observed when *V. nugundo and* chloramphenicol, or and *G. superba* and chloramphenicol were combined. Synergism was observed on *C. albicans*. The factors responsible for the synergistic effect were not known during the study especially as *C. albicans* is a fungus. However, since *C. albicans* is gram positive,[31] the mode of action of the plant extracts might be similar to that of chloramphenicol on gram positive bacteria. Elsewhere, synergistic effect was observed for the combination of garlic and omeprazole against *Helicobacter pylori* and none or even antagonistic effect was observed between garlic and amoxycillin, clarithromycin or metronidazole.[36] This indicates that synergism between a medical plant and a chemotherapeutic agent may be selective. That is, there may be synergism between one plant and a particular chemotherapeutic agent, and may not be so in another combination.

There was antagonism observed between the two test plants in their combined effect on *E. coli* and *S. typhi* as exhibited by the MIC of the plant extracts. The combined MIC of the plant extracts showed neither antagonism nor synergism on *S. aureus* and *C. albicans*. Significant differences ($P < 0.05$) in the MIC of the plant extracts (VN, GS and VN + GS) was observed. Least significant difference (LSD) test showed that there was significant difference ($P < 0.05, 0.01$) between VN and GS and between VN and VN + GS, while there

Table 3: The minimum inhibitory concentation (MIC) of *V. nugundo* (VN) and *G. superba*, (GS) singly and in combination on the test organisms

Test organisms	MIC (µg)		
	VN	GS	VN + GS
Escherichia coli	6.15	4.22	4.22
Salmonella typhi	11.40	5.15	5.15
Staphylococcus aureus	50	5.25	3.12
Candida albicans	50	50	25

was no significant difference ($p > 0.05$) between GS and VN + GS. Thus, *G. superba* (GS) with smaller MIC against the test organisms was more active than *V. nugundo* (VN).

We conclude that *V. nugundo* (VN) and *G. superba* (GS) might individually be very effective against *C. albicans* infections. Chloramphenicol alone has no effect on *C. albicans,* but its combination with either VN or GS might produce a synergistic effect on *C. albicans* infections and on gram positive bacterial infections. There is need for further research in this aspect. Antagonism among herbs should be further studied to assist traditional herbalists who always combine them in treatment.

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Chromatographic Evaluation of Terpenoids of some medicinally important barks

Udaysing Hari Patil*[1] and Dattatraya Krishna Gaikwad[2]

[1]*Department of Botany, Bhogawati Mahavidyalaya, Kurukali, Tal- Karveer, Dist-Kolhapur [MS] India-416001*
[2]*Department of Botany, Shivaji University Kolhapur [MS] India-416004*

ABSTRACT

Thin layer Chromatographic analysis of bark of *Anogeissus latifolia, Crataeva religiosa, Pterocarpus marsupium* and *Terminalia arjuna* was carried with respect to the terpenoid pool of the plants. The three bark samples (Apical bark, middle bark and mature inner bark on main trunk) of each plant were analyzed and maximum terpenoids were recorded in the apical bark of *Terminalia arjuna* stem bark.

Key words: Chromatographic analysis, terpenoids, *Anogeissus latifolia, Crataeva religiosa, Pterocarpus marsupium, Terminalia arjuna*

INTRODUCTION

Terpenoids constitutes the largest class of biologically active product and play defensive role against predators, pathogens, and competitors, maintaining antagonistic and beneficial interactions among organisms.[1] The important classes of terpenoids present in plants are the volatile essential oils, triterpenoids, steroids and carotenoids.[2] These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure is $C_{10}H_{16}$ and they are called terpenes. The isoprene unit, which can build upon itself in various ways, is a five-carbon molecule. The single isoprene unit, therefore, represents the most basic class of terpenes, the hemiterpenes. An isoprene unit bonded with a second isoprene is the defining characteristic of terpene, which is also a monoterpene (C_{10}). Their general chemical structure occur as diterpenes, triterpenes and tetraterpenes (C_{20}, C_{30} and C_{40}), as well as hemiterpenes (C_5) and sequiterpenes (C_{15}). When the compounds contain additional elements, usually oxygen, they are termed as terpenoids. While sesquiterpenes contain three isoprene units (C_{15}), diterpenes (C_{20}) and triterpenes (C_{30}) contain two and three terpene units, respectively. Tetraterpenes consist of four terpene units and polyterpenes are those terpenes containing more than four terpene units (i.e., more than eight isoprene units).[3]

Address for correspondence:
E-mail: superoxide2311@gmail.com

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Plant *A. latifolia* has been used to treat epileptic fits and cough.[4] *Anogeissus latifolia* bark shows wound healing potential[5], antiulcer potential[6] and hepatoprotective activity.[7] Bark of *Crataeva religiosa* is especially useful in kidney bladder stones, fever, vomiting and gastric irritation[8]. The bark posses antimycotic activity[9], antinocicepative property[10], anti-inflammatory activity[11] and anti-oxaluric effects[12]. *Pterocarpus marsupium* is useful in diabetic anemia[13]. Bark is useful in vitiated condition of *kapha* and *pitta*, elephantiasis, erysipelas, urethrorrhea, rectalgia, opthalmopathy, hemorrhages, dysentry, cough and grayness of hair.[14,15] *Terminalia arjuna* is known for its use in heart trouble[16] and wound healing potential.[17] This plant is effective in many cardiac disorders like angina, myocardial infarction, hypertension, hypercholesteremia, cardiac arrest etc.[18-19].

MATERIAL AND METHODS

The plant material was collected from the Radhanagari, Kagal, Panahala and adjoining areas of the Kolhapur district. Plant material was separated in to three categories as 1) Bark harvested from the apical branches from the top of the tree regarded as Young bark, 2) Bark harvested from branches, 10-20 feet away from the apical branches regarded as middle bark, and 3) Bark harvested from main trunk of the tree regarded as mature bark Different bark samples were sun dried and then in oven maintained at 50°C. Methanol extract of the powdered bark sample was used for the TLC analysis. Terpenoid were separated on TLC plates according to the method described by Wagner and Bladt.[20]

RESULTS AND DISCUSSION

Results of the present investigations are shown in Figure 1 and Table 1. From the figure it is clear that all plant samples showing difference in the terpenoid composition. Young and middle bark of *A. latifolia* shows similar terpenoid bands (8) while, mature bark contain less number (5) of bands as compare to young and middle. In case *C. religiosa,* young and middle bark contains equal number (8) of terpenoid bands but with different R*f* values whereas mature bark contains one additional terpenoid band than young and middle bark with different R*f* values. In case of *P.marsupium*, middle bark contains maximum number of terpenoid bands (12) than young (11) and mature bark (9). Young bark of *Terminalia arjuna* contains the maximum number of terpenoid bands (19) than the middle (15) and mature bark (14). Thus, among all the bark samples studied, *Terminalia arjuna* constitutes maximum number of Terpenoids than the other three plants. Kessler and Baldwin[21] have shown role of terpenes in plant defense. A terpenoid constituent, capsaicin, has a wide range of biological activities in humans, affecting the nervous, cardiovascular and digestive systems[22] as well as an analgesic.[23] Kwon-Ghung and Bennette,[24] studied the pathogenic aspects of *Aspergillus*, responsible for most human systemic infections. Matura *et al*.[25] investigated the role of some terpens play as causative agents of contact dermatitis and fragrance allergies. Our results indicate that, there is decrease in terpenoid pool with ageing of the bark. There are different terpenoids present among the three bark

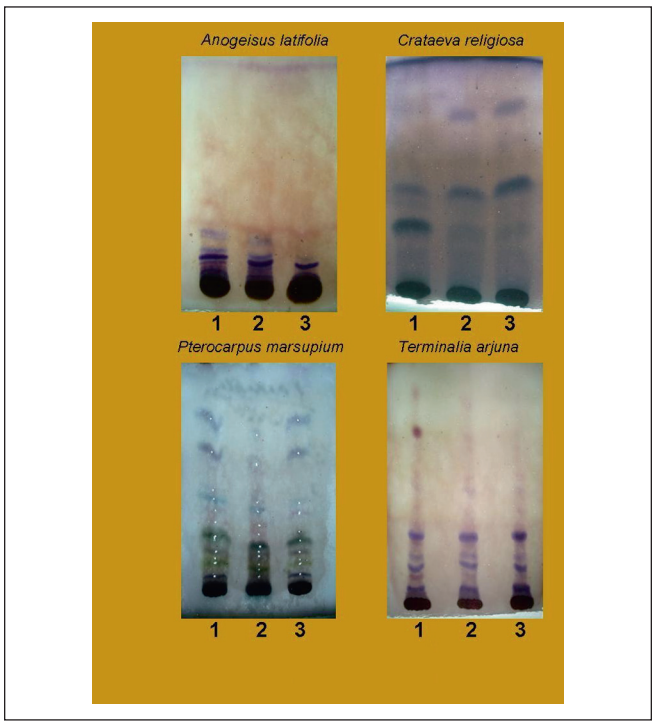

Figure 1: Chromatographic analysis of different bark samples. 1: Apical bark; 2: Middle bark and 3: Mature inner bark

Table 1: Rf values of Terpenoids from barks of *A. latifolia, C. religiosa, P. marsupium* and *T. arjuna*

Samples ↓ / Band →	i.	ii.	iii.	iv.	v.	vi.	vii.	viii.	ix.	x.	xi.	xii.	xiii.	xiv.	xv.	xvi.	xvii.	xviii.	xix.
AY	0.05	0.06	0.08	0.10	0.12	0.15	0.20	0.23	–	–	–	–	–	–	–	–	–	–	–
Am	0.05	0.06	0.08	0.10	0.12	0.15	0.20	0.23	–	–	–	–	–	–	–	–	–	–	–
AM	0.06	0.07	0.08	0.10	0.14	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CY	0.06	0.10	0.12	0.18	0.21	0.27	0.30	0.40	–	–	–	–	–	–	–	–	–	–	–
Cm	0.06	0.08	0.11	0.15	0.19	0.21	0.24	0.27	–	–	–	–	–	–	–	–	–	–	–
CM	0.06	0.08	0.10	0.14	0.16	0.21	0.27	0.32	0.35	–	–	–	–	–	–	–	–	–	–
PY	0.06	0.11	0.13	0.16	0.21	0.23	0.28	0.33	0.38	0.42	0.57	0.72	–	–	–	–	–	–	–
Pm	0.06	0.08	0.11	0.12	0.15	0.16	0.18	0.23	0.27	0.33	0.37	0.53	0.65	–	–	–	–	–	–
PM	0.06	0.10	0.11	0.16	0.20	0.31	0.35	0.57	0.71	–	–	–	–	–	–	–	–	–	–
TY	0.02	0.03	0.05	0.08	0.10	0.13	0.16	0.18	0.20	0.21	0.24	0.26	0.30	0.37	0.45	0.50	0.60	0.65	0.82
Tm	0.02	0.03	0.05	0.09	0.11	0.14	0.019	0.22	0.26	0.31	0.34	0.43	0.49	0.64	0.76	–	–	–	–
TM	0.02	0.04	0.05	0.08	0.10	0.13	0.017	0.22	0.26	0.30	0.36	0.43	0.49	0.60	–	–	–	–	

A- Anogeisus latfolia; C- Crataeva religiosa; P- Pterocarpus marsupium and T- *Terminalia arjuna*
Y- young bark; m- middle bark and M- mature inner bark

samples of the same plant and it is evidenced from the chromatogram shown in the Figure 1. The diversity of these bioactive compounds might have formed one of the active principle(s) in theses crude drugs which have been reflected through their various pharmacological activities and claims their use in various Ayurvedic remedies since ancient times.

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Evaluation of *Invitro* anthelmintic activities of *Brassica nigra, Ocimum basilicum* and *Rumex abyssinicus*

Saleem N Basha[1]*, R Rekha[2], Sirak Saleh[1] and Samuel Yemane[1]

[1]Department of Pharmaceutics, School of Pharmacy, Asmara College of Health Sciences, Asmara, Eritrea, NE Africa.
[2]Department of Pharmacognosy, SRM College of Pharmacy, Kattangulathur, Chennai, Tamilnadu, India.

ABSTRACT

Helminthes infections are now being recognized as the cause of many acute as well as chronic illnesses in human beings and in cattle, particularly in tropical and subtropical areas. The development of anthelmintic resistance and the high cost of conventional anthelmintic drugs led to evaluation of herbal remedies as alternative anthelmintic for the control of these parasites. In the current study, *in vitro* experiments were conducted to determine the possible anthelmintic effects of crude aqueous and methanolic extracts of *Brassica nigra (seeds), Ocimum basilicum (whole plant)* and *Rumex abyssinicus (roots)* in a dose of 20 and 40mg/ml against Eritrean adult earthworm *Pheretima posthuma* to justify the traditional claim using mebendazole as a reference standard. From the results, the paralysis and death time of worms in all extracts was found to be dose dependent and the potent anthelmintic activity was observed in aqueous extracts in comparison to methanolic extracts. Among the three plants *Ocimum basilicum* showed a potent anthelmintic activity with least paralysis time and death time, whereas *Rumex abyssinicus* extracts showed an intermediate activity and *Brassica nigra* has taken long time for death of worms. All the values were found to be statistically significant with P value of 0.00 using one way ANOVA. Based on the findings it may be concluded that *Brassica nigra, Ocimum basilicum* and *Rumex abyssinicus* possess varying degree of anthelmintic activity and also justifies its folklore claims for potential anthelmintic property.

Key words: Anthelmintic activity, *Brassica nigra, Ocimum basilicum, Rumex abyssinicus.*

INTRODUCTION

Helminthes are recognized as a major constraint to livestock production throughout the tropics and elsewhere and are considered important in causing enormous economic losses through morbidity and mortality in livestock. As most of the developing countries of the world lie in tropical and subtropical region, warm and humid climatic conditions in the tropic/subtropics provide favorable environment for development of worm eggs to infective larvae almost throughout the year.[1]

Among the parasitic diseases, gastro-intestinal (g/I) nematodes such as *Haemonchus contortus, Trichostrnngylus spp., Cooperia spp, Oesophagostomum columbianum, Trichuris spp.* and *Strongyloides papillosus* are most common cause. This group of gastrointestinal nematodes is associated with anaemia and gastroenteritis resulting loss of body weight, stunted growth, diarrhoea etc. that greatly hamper the normal growth and production of goats. Thus parasite problem is unquestionably being a major limiting factor in the improvement of livestock production.[2] *Haemonchus contortus* is a highly pathogenic helminthes of small ruminants in all age groups, and is one of the top 10 constraints of sheep and goat production in East Africa.[3]

Helminthes infections are also among the most common infections in human, affecting a large proportion of the world's population in developing countries and produce a global burden of disease and contribute to the prevalence of malnutrition, anaemia, eosinophilia, and pneumonia which more often physically impair their hosts than kill them.[4] Control of gastrointestinal Helminthes infections in the livestock relies mainly on the use of anthelmintic in combination with farm management.[5]

Various problems have been evolved with chemotherapeutic control practices such as parasites are developing resistance

Address for correspondence:
E-mail: nsaleem_basha@rediffmail.com

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to several families of chemical anthelmintics,[6] chemical residues and toxicity problems,[7] un-economical and non-availability of drugs in remote areas. Furthermore, it has been recognized recently that anthelmintic substances having considerable toxicity to human beings are present in foods derived from livestock, posing a serious threat to human health.[8]

For these various reasons, interest in the screening of medicinal plants for their anthelmintic activity remains of great scientific significance despite extensive use of synthetic chemicals in modern clinical practices all over the world. In early times of the eldest human societies (Babylonia, Egypt, Greece, and S. Klimpel Rome), but also stretching from the early medieval centuries until the nineteenth century, plant diets were used to control parasites inside the house, in farm animals, as well as in humans based more on belief than on knowledge.[9] The plant kingdom is known to provide a rich source of botanical anthelmintics, antibacterials and insecticides.[10] A number of medicinal plants have been used to treat parasitic infections in man and animals.[11] However, their scientific evaluation as compared to commercial anthelmintics is limited. These factors paved the way for herbal remedies as alternative anthelmintic. World Health Organization estimates that 80% of people in developing countries rely on traditional medicines.[12]

In Africa and other developing countries, most people depend on traditional herbal medicine for health needs, and these herbal remedies are usually safer than active compounds isolated from plants.[13] Plant-derived anthelmintic products have the advantage of being more biodegradable and environmentally friendly.[14] A larger number of plants are naturally available in the Eritrea, which possess narrow or broad spectrum anthelmintic activities. Therefore the phytochemical analyses of naturally available plants and control anthelmintic trials along with contemporary knowledge of parasite control strategies may offer new opportunities for effective and economical control of parasitic diseases.

Based on information obtained from ethno-medicinal survey and on information obtained from herdsmen and pastoralists, the plants *"Brassica nigra"*, *"Ocimum basilicum"* and *"Rumex abyssinicus"* were selected. A review of the literature also revealed that the anthelmintic activity of these plants has not been subjected to scientific evaluation for its anthelmintic activity. Hence, the present study was carried out on aqueous and methanolic extracts of these three medicinal plants against Eritrean adult earthworm *"Pheretima posthuma"* to justify its folkfore claim also aimed to make documentation and validation of these plants in a scientific approach in Eritrea. Recognizing the value of this indigenous knowledge empowers livestock owners to attempt to solve their animal health problems in a cost effective way.

MATERIALS AND METHODS

Plant material

The 3 medicinal plant materials i.e., Seeds of *Brassica nigra (Brassicaceae),* English name: Black mustard and Vernacular name: Adri*,* Whole plant of *Ocimum basilicum (Lamiaceae),* English name: Sweet basil and Vernacular name: Seseg and Roots of *Rumex abyssinicus (Polygonaceae),* English name: Spanish rhubarb and Vernacular name: Mokmoko were procured from the local market/field (Medeber), Asmara, Eritrea in April 2010. The collected plant materials were authenticated by the Taxonomist Dr. Gebrehiwet Medihanie, Department of Biology, Eritrean institute of technology (EIT), Mai Nefhi, Eritrea and a voucher specimen was numbered and kept in our research laboratory for further reference.

Extract preparation

All collected plant materials were thoroughly cleaned with water to remove adulterants and dried under shade at a well ventilated place for five days at 35-40°C until it became grindable. The dried materials were ground with the help of electric grinder and preserved separately for further extraction with respective solvents.

Aqueous extracts preparation

Powdered materials of *Brassica nigra* (60 g), *Ocimum basilicum* (20 g) and *Rumex abyssinicus* (50 g) were extracted separately in soxhlet apparatus using distilled water at 50°C. then the extract was filtered using What man No 1 filter paper, the clear filtrate obtained was concentrated by a rotary evaporator at a temperature not exceeding 40°C under reduced pressure, lyophilized and the resulting powder was stored at 4°C until use, and dissolved in solution of 0.1% Tween 80 in a normal saline on the day of the experiment to prepare stock solution and different test dilutions for the purpose of evaluating anthelmintic activity. The percentage of extraction was calculated by using the following formula: percentage of extraction = weight of the extract (gram)/ weight of the plant material (gram) × 100.

Methanol extracts preparation

Powdered materials of *Brassica nigra* (50 g), *Ocimum basilicum* (20 g), and *Rumex abyssinicus* (60 g) were used for extraction. Each plant materials were separately soaked in sufficient amount of methanol by cold maceration at room temperature for a total of 3 days. After that the filtrates were collected through a What man No 1 filter paper under vacuum pressure and the plant materials re-soaked twice. The combined filtrates of each plant material was concentrated in a rotary evaporator at 40°C under reduced pressure to yield a thick and dark coloured crude extract, lyophilized and the resulting powder was stored at 4°C until use and

dissolved in a solution of 0.1%Tween 80 in normal saline on the day of the experiment to prepare stock solution and different test dilutions for the purpose of evaluating anthelmintic activity. The percentage of extraction was calculated by using the following formula: percentage of extraction = weight of the extract (gram)/ weight of the plant material (gram) × 100.

Preliminary phytochemical screening

Standard methods[15] were used for preliminary phytochemical screening of methanolic and aqueous extracts to know the nature of phyto constituents present in it.

Evaluation of anthelmintic activity

Drugs

The aqueous and methanolic extracts of *Brassica nigra, Ocimum basilicum* & *Rumex abyssinicus* were tested in two doses i.e., 20 mg/ml & 40 mg/ml in each group. Tween 80 (0.1%) in normal saline was used as control. The standard drug Mebendazole was obtained from Azel Pharmaceuticals, Keren as a gift sample for comparative study with aqueous and methanolic extracts. All other chemicals and solvents used were of analytical grade and obtained locally.

Experimental animal model

Healthy adult Eritrean earthworms, *Pheretima posthuma*, due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings[16] were used in the present study. Because of easy availability, earthworms have been widely used for the initial evaluation of anthelmintic compounds *in vitro*.

Worm collection and authentication

Eritrean adult earthworms (Pheretima posthuma; Annelid, Megascolecidae) collected from moist soil of "Maitemenay" around "Enda- kirbit" of Asmara (Eritrea) and washed with normal saline to remove all extraneous matters. All earthworms were of approximately of 4-8 cm in length and 0.1-0.2 cm in width was used for all the experimental protocol. These Earth worms were identified and authenticated by Mrs. Ghimja, Parasitologist, Head, School of Allied Health Professions, Asmara College of Health Sciences, Asmara, Eritrea.

Grouping of worms

Four groups of approximately equal size earthworms consisting of three earthworms in each group were used for the present study. **Group first** serve as control, receive only Tween 80 (0.1%) in normal saline, **Group second** serve as test-1, receive Methanolic extracts, **Group third** serve as test-2, receive aqueous extract and **Group four** serve as standard, receive standard drug Mebendazole of different concentration.

Anthelmintic assay

Screening of plants for their anthelmintic activity has multiple objectives. These include:

1. Validation of the claims of the farmers using different plants for anthelmintic purposes using standard parasitological procedures
2. Exploring the possibilities of discovering new plants with anthelmintic properties.

The anthelmintic activity was evaluated as per the method of Dash et al[17] with slight modification. The extracts were suspended in Tween 80 (0.1%) in normal saline. All the drugs and extracts were prepared freshly before starting the experiment. Twelve groups of six earthworms each were released into 10 ml of desired formulation as follows; vehicle (Tween 80 (0.1%) in normal saline, Mebendazole (20 and 40 mg/ml), methanol and aqueous extract (20 and 40 mg/ml, each) in Tween 80 (0.1 %) in normal saline.

Observation was made for the time taken to paralysis and death of individual worms up to 4 hrs of the test period. Time for paralysis was noted when no movement of any sort could be observed except when the worms were shaken vigorously. Time for death of worms were recorded after ascertaining that the worms neither moved when shaken vigorously nor when dipped in warm water (50°C). Death was concluded when the worms lost their motility followed with fading away of their body colours. All the results were expressed as a Mean ± SEM of six animals in each group.

Statistical analysis

All the data's were subjected to one way ANOVAs analysis for calculating *P* value using the SPSS software Version 18. Results with $p < 0.05$ were considered to be statistically significant.

RESULTS

In the present work, aqueous and methanolic extracts of selected plants used in traditional medicine in Eritrea were tested for their anthelmintic activity. The percentage yield of aqueous extracts of *Brassica nigra, Ocimum basilicum* & *Rumex abyssinicus* were found to be 1.4, 4.5, 5.54% w/w respectively with reference to the dried plant material. Similarly the percentage yield of Methanolic extracts of *Brassica nigra, Ocimum basilicum* & *Rumex abyssinicus* were found to be 6.52, 10.8, 10.73% w/w respectively with reference to the dried plant material. Preliminary phytochemical screenings on these extracts revealed the presence of flavonoids, alkaloids, saponins, carbohydrates and tannins in the extracts.

From Table 1 and Table 2, it is very clear that methanol and aqueous extracts showed better anthelmintic activity

when compared with standard drug at the same concentration. Vehicle worms were alive up to above 12 hrs of observation. From the observations made, the paralysis and death of worms in different doses were found to be dose dependent. Among extracts, potent anthelmintic activity was observed in case of aqueous extract than the methanolic extracts.

From the results, it was observed that Ocimum *basilicum* showed a potent anthelmintic activity from all the aqueous and methanolic extracts. Aqueous and methanolic *Rumex abyssinicus* extracts shows an intermediate activity. Whereas extracts of *Brassica nigra* took longer time to bring death of worms. Even if this was the case these effects are even comparable with the paralysis and death time of Mebendazole.

DISCUSSION

This study revealed that crude extracts of *O. basilicum, B. nigra* and *R. abyssinicus* exhibited strong *in vitro* anthelmintic activity against the model organism. Based on the preliminary phytochemical screening crude extracts of *Brassica nigra, Ocimum basilicum* and *Rumex abyssinicus* revealed presence of tannins as one of the chemical constituent, which are known for their antimicrobial activity and also have their application as anthelmintic.[18] Chemically tannins are polyphenolic compounds. Some synthetic phenolic anthelmintics e.g. niclosamide, oxyclozanide and bithionol are shown to interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation. Another possible anthelmintic effect of tannins is that they can bind to free proteins in the gastrointestinal tract of host animal or glycoprotein on the cuticle of the parasite and cause death.[19,20] Presence of Tannins may be responsible for anthelmintic activities of plant extracts.

Mebendazole, a benzimidazole drug that acts by interfering with carbohydrate metabolism and inhibiting polymerization of microtubules. The function of the anthelmintic drugs like Mebendazole is to cause paralysis of worms. So, that they are expelled in the faeces of man and animals.[21] The extracts not only demonstrated paralysis of worms, also caused death of the worms. Hence our study has shown that, all the extracts have significantly determined anthelmintic activity. Statistical analysis reveals that significant in vitro anthelmintic activity ($P = 0.00$) was observed between the concentrations and time of exposure of all the extracts. The present investigation indicated that all the plant extracts has antihelminthic effects against Eritrean adult earth worm in vitro at the various concentrations tested.

CONCLUSION

The experimental evidence obtained in the laboratory model could provide a rationale for the folklore claims of *Brassica nigra, Ocimum basilicum* & *Rumex abyssinicus* for its potential anthelmintic property. These analyses have revealed for the

Table 1: Anthelmintic activities of methanolic extracts

Test sample	Doses in mg/ml	Time taken for paralysis (P) and death (D) in minutes	
		P	D
Brassica nigra	40	146.33 ± 0.72	189 ± 1.40
	20	156.67 ± 0.719	203.67 ± 1.784
Ocimum basilicum	40	22.67 ± 0.27	67.33 ± 4.12
	20	96.67 ± 1.03	150.67 ± 0.54
Rumex abyssinicus	40	29.67 ± 0.98	57.33 ± 0.27
	20	58 ± 1.25	91.67 ± 1.9
Mebendazole (Standard)	40	73.3 ± 0.72	98.67 ± 0.98
	20	101.33 ± 0.72	121 ± 0.94

The value paralysis time (P) and death time (D) refer the mean ± SEM

Table 2: Anthelmintic activities of aqueous extracts

Test sample	Doses in mg/ml	Time taken for paralysis (P) and death (D) in minutes	
		P	D
Brassica nigra	40	17 ± 0.00	36.67 ± 0.54
	20	23.66 ± 0.271	41.33 ± 1.65
Ocimum basilicum	40	5 ± 0.54	8 ± 0.72
	20	8 ± 0.47	12.3 ± 0.72
Rumex abyssinicus	40	12 ± 0.00	25.33 ± 0.00
	20	36.67 ± 0.54	57.67 ± 0.35
Mebendazole (Standard)	40	73.3 ± 0.72	98.67 ± 0.98
	20	101.33 ± 0.72	121 ± 0.94

The value paralysis time (P) and death time (D) refer the mean ± SEM

first time that the components present in the solvent extracts of *O. basilicum, B. nigra* and *R. abyssinicus* have anthelmintic activity. These results strongly indicate that plant extracts derived from O. basilicum, B. nigra and R. abyssinicus contain promising bioactive compounds that might be useful in the control of helminth infections by interrupting the worms' life cycle and preventing their growth. Since the motility of worms is also affected, it may be possible that these plant extracts also act on worm muscles and paralyse them. Remarkably, none of these selected plants have been previously tested. Our data reinforces the existing knowledge and the regular use of plants by herdsmen and pastoralists for the treatment of worm infections. So, it may be suggested that those 3 plants with strong wormicidal activity could provide alternatives in the control of helminthic infections. The high anthelmintic activity observed make these plants good candidates for isolation of anti-helmintic compounds which could serve as new lead structures for drug development and offers an opportunity for developing alternatives to rather expensive drugs.

In future studies, we will isolate active compounds and investigate their mode of action. Additionally we are looking into safety aspects to determine the feasibility of developing effective and safe drug preparations for the treatment of helminth infections.

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