# Development of Pharmacognostical Parameters and Estimation of β-sitosterol using HPTLC in Roots of *Gmelina arborea* Roxb

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## ABSTRACT

This paper deals with the detailed pharmacognostical evaluation of roots of *Gmelina arborea* Roxb. (Verbenaceae), a highly valued plant in Ayurveda as a bitter tonic, stomachic, laxative and useful in fever, indigestion, anasarca and skin problems. It has been used as an ingredient in many preparations like Dashmoola, Panchmoola di kwatha and Chyawanprasha. Chemical constituents of *G. arborea* roots have been studied extensively which include lignans, flavonoids, alkaloids and various phenyl propanoid glycosides but pharmacognosy of roots has not been reported. The main objective of the present work is to develop detailed pharmacognostical and physicochemical parameters recommended by World Health Organization (WHO) and various pharmacopoeias for the roots, as this has not been reported so far for the roots of the plant. Preliminary analysis showed the presence of phenolics and flavonoids, which led us to estimate total phenolics in roots. The present study also focuses on development of high-performance thin-layer chromatographic (HPTLC) method for the quantitative analysis of roots using β-sitosterol as a chemical marker. Major microscopic features of roots are lignified cork, presence of oil globules, scattered stone cell islets in secondary cortex and pitted wood elements. Phytochemical screening reveals the presence of flavonoids, lignans, sterols, tannins, carbohydrate, coumarins and alkaloids. Total phenolics were found 1.89% w/w in roots and β-sitosterol was found 0.120 ± 0.018% in methanolic extract of the roots by the developed HPTLC method. These findings will aid in the standardization of one of the major roots used in a well known Ayurvedic formulation, Dashmoola. Validated HPTLC method developed can be used as a tool for standardization of roots in different formulations using β-sitosterol as a marker.

**Key words:** β-sitosterol, Dashmoola, *G. arborea*, HPTLC, physicochemical parameters

## INTRODUCTION

Many medicinal plants, traditionally used for thousands of years, are present in a group of herbal preparations of the Indian traditional health care system (Ayurveda) and proposed for their interesting multilevel activities. Amongst the medicinal plants used in Ayurvedic preparations for their therapeutic action, some have been thoroughly investigated and some of are still to be explored. One such plant, *Gmelina arborea* Roxb. (Verbenaceae) is well reputed for its multilevel activities in Ayurveda. It is also known as Gambhari, Krishna Vrinlaka, and Shriparni in Sanskrit and other biological names are *Gmelina indica* Burm. F., *Gmelina rheedii* and *Premna arborea*. It is distributed throughout India, Ceylon, Malayan and Philippine Islands. In India, *G. arborea* occurs extensively in the sub-Himalayan tracts, Assam, Northern West Bengal, South Bihar and Orissa and can be planted elsewhere on a large scale.[1,2,3] The roots are used in many well-known Ayurvedic preparations like Dashmoola and Chyawanprasha. It belongs to group of five major roots (Mahat panchmula) of Dashmula di kvatha which is reported to be used in chronic fever, rheumatic affection, haemorrhages, urinary tract infection, anuria and dysuria. The main preparations are Shriparnaadi kwatha and Panchmulaadi kwatha.[4] Further, *Gmelina arborea* and Dashmoola found widely are used in many herbal products like abana, Geriforte, Chyawanprasha, arvindasav, etc.[5] Roots are useful in hallucinations, and for the treatment of piles, abdominal pains, burning sensations and urinary discharges.[6-8] It has been found useful in indigestions, fever,

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and anasarca in the form of infusion or decoction. It is also given as an appetite stimulant, anthelmintic and as galactogogue with liquorice, sugar and honey.[6,8-11] Roots have been reported to possess anti malarial[12] and cardiovascular activity.[13] Chemical constituents of *G. arborea* include lignans[14] flavonoids,[15] alkaloids[16] and various phenyl propanoid glycosides[17] mainly found in the heartwood and bark of the plant. Roots of this plant are highly valued in the traditional system of medicine and the pharmacological and chemical composition of roots has been well explored. However, to our knowledge pharmacognostical and physicochemical parameters have not been developed so far for the roots. Further, heartwood, stem bark and roots have been reported to contain many terpenoids, flavonoids and lignans but a validated analytical method for the estimation of marker compound has not been developed for the roots of this plant. Hence, the present study focuses on the evaluation of physicochemical parameters to develop the monograph detail along with the validated HPTLC method for quantification of β-sitosterol in the roots. This method forms the basis of standardization of the formulation containing roots of *G. arborea* using β-sitosterol as a marker compound.

## MATERIALS AND METHODS

### Plant Material

Fresh mature roots were collected from fully-grown trees from fields near the outskirts of Mahesana, Gujarat in September, 2006. The authenticity was established by comparing its morphological and microscopical characters with the available literature[3] and by a taxonomist of Gujarat Ayurveda University, Jamnagar. Roots were dried under shade, powdered and then stored in air tight containers for further use. Voucher specimen (LM 16) was deposited at Dept. of Pharmacognosy, L. M. College of Pharmacy, Ahmedabad.

### Chemicals and instruments

Charged Coupled Device (CCD, Lawrence and Mayo) camera attached with compound microscope, solvents viz. petroleum ether, benzene, chloroform, acetone, ethanol (95%), n-butanol, ethyl acetate and various reagents like phloroglucinol, glycerine, hydrochloric acid, chloral hydrate were procured from India Scientific, India. Folin Ciocaluteu reagent was procured from Sigma Chemical Co. (St. Louis, MO, USA).

### Macroscopic and microscopic observations

Roots were studied for their detailed morphological characters and freehand sections of root were taken and stained with a number of reagents for histochemical examination. All the observations of the microscopical study of roots and powder were made and recorded with the help of a special CCD camera attached with the microscope.

### Physicochemical analysis

Physicochemical analysis was performed by the determination of different ash values, extractive values, loss on drying according to the official methods prescribed[18] and the WHO guidelines on quality control methods for medicinal plant materials.[19] The fluorescence properties were studied under ultra violet (UV) light adopting the method described by Kokoshi[20] and Chase & Pratt.[21] A small quantity of root powder was placed on a clean microscopic slide and one to two drops of freshly prepared reagent solution was added, followed by gentle mixing. The behaviour of the sample with different chemical reagents was studied and fluorescence characters were observed on short and long UV light after 1-2 minutes.

### Preliminary phytochemical screening

A known quantity of powdered roots was extracted sequentially with petroleum ether, chloroform, ethyl acetate and methanol and then left for the maceration in water for 12 hours. Preliminary phytochemical screening was carried out using standard procedures described by Harborne[22] by subjecting different fractions to tests separately for the presence of various phytoconstituents like alkaloids, flavonoids, sterols, saponins, lignans, coumarins, carbohydrates and tannins.

### Estimation of total phenolics[23]

One g of air-dried root powder was extracted with 100 ml methanol by maceration for 24 hours and filtered. The final volume of the filtrate was adjusted to 100 ml using methanol. Five ml of this extract was diluted with an equal volume of methanol and was used for the estimation of phenols. To 10 ml of the methanolic extract, 10 ml of distilled water and 1.5 ml of diluted (1:2) Folin Ciocaulteu reagent were added and the mixture was kept for 5 minutes. After adding 4 ml of 20% $Na_2CO_3$ solution, the final volume was adjusted to 25 ml using distilled water. The absorbance was measured at 765 nm at an interval of 30 minutes up to 2 hours using distilled water as a blank. The data was compared with similarly prepared set of standard substance gallic acid in a concentration range of 50 µg to 300 µg per 25 ml. The total phenol content C was measured using the following formula:

$$\mathbf{C = A \times 282.6 - 8.451} \quad (A = \text{absorbance})$$

### Quantification of β-sitosterol in roots of *G. arborea* by HPTLC

#### *Instruments*

Camag Linomat V (Semi automatic spotting device), Hamilton 100µl HPTLC syringe, Camag twin trough

chambers (20 × 10 cm), Camag TLC scanner 3, 120311, Camag CATS 4 integration software, Camag Reprostar – 3

### *Materials and chemicals*

Precoated silica gel 60 $F_{254}$ aluminium plates (Merck, Darmstadt, Germany) with a thickness of 0.1 mm, 10 × 10 cm, ethyl acetate, methanol, toluene, solvent ether, chloroform, distilled water, anisaldehyde sulphuric acid (0.5% anisaldehyde in sulphuric acid in methanol). All the reagents used were of analytical grade.

### *Preparation of test samples and standard solutions for HPTLC*

HPTLC analysis was performed to confirm the presence of β-sitosterol by co chromatography with available reference standard of β-sitosterol procured from Sigma, USA. Five grams of accurately-weighed root powder was extracted with methanol (2 × 50 ml) on a boiling water bath by refluxing for 1 hour. Combined filtrates were evaporated to dryness and used for preparation of stock solution of extract for spotting on plate.

### *Calibration curve of ß-sitosterol and development of chromatogram*

Accurately weighed 10 mg of standard β-sitosterol was dissolved in methanol to prepare a solution of 1 mg/ml strength. Graded concentration of standard solution (1 mg/ml) in 0.5, 1, 2, 5 and 7.5 µl volume was applied on a precoated TLC silica gel 60 $F_{254}$ plate so that the concentration of β-sitosterol was in the range of 500, 1000, 2000, 5000 and 7500 ng/spot. Two spots of methanolic extract (2 µl) were spotted and the plate was developed in a mobile phase, toluene: methanol (9.4:0.6) and dried using a hair dryer on hot mode for 5 minutes. After complete removal of the solvent from the plate, it was derivatized by 0.5% anisaldehyde sulphuric acid reagent followed by heating at 110 °C for 10 minutes and scanned at 523 nm. The calibration curve was obtained by plotting the area versus concentration of each peak corresponding to the respective spot.

### *Validation of developed HPTLC method*

The developed method was validated in terms of linearity, precision, repeatability, accuracy, specificity, limit of detection and limit of quantification.

## RESULTS

### Macroscopic studies of roots of *G. arborea*

Roots occur as segments 5-15 cm in length and 3-20 mm in diameter, cylindrical to tapering, with secondary and tertiary branches and bearing slender and tapering rootlets. External surface is uneven, dull, rough and longitudinally wrinkled. It occasionally shows rootlet scars on the larger pieces, with some exfoliation of the bark in small areas revealing the paler wood beneath. The freshly fractured surface shows a thin layer of greyish yellow bark, and the pale wood constituted about 80% of the radius (Figure 1). Dried pieces of mature root bark, curved and channelled, thinner ones forming single quills, external surface was rugged due to presence of vertical cracks, ridges, fissures and numerous lenticels. Wood was light to moderately heavy, hard, strong, elastic and lustrous with a smooth feel. Fracture was somewhat tough in bark, brittle and predominant in woody portion.

### Organoleptic properties

Externally light brown to greyish yellow and yellowish white wood. Mature root bark when fresh was yellowish in colour. Root odour was indistinct and taste was mucilaginous and sweetish with slight bitterness.

### Microscopic studies of roots of *G. arborea*

Microscopic studies were carried out by studying different histological characters of the transverse as well as longitudinal sections of roots and powdered drug. Observations from


**Figure 1:** Plant photograph and morphology of roots of *G. arborea*

the unstained and stained slides were studied using various reagents viz. phloroglucinol and concentrated hydrochloric acid for visualizing elements with lignin thickening, dilute Iodine solution for starch and ruthenium red for mucilage.

## Transverse section of Root

A portion of a transverse section from the middle of a soaked root pieces was found to show the following characteristics (Figure 2).

Tangentially elongated rectangular and lignified cork cells were sometimes broken towards upper layers; there were 15-20 layers in young roots and 30-35 layers in mature roots. Cork was followed by indistinct phellogen. The secondary cortex was composed of several rows of tangentially elongated thin wall parenchymatous cells, densely filled with starch grains and oil globules. Scattered resin ducts and stone cells were either solitary or in groups of two to four cells, occasionally pitted and highly thickened (60-100 μ). Occasional presence of prisms of calcium oxalate crystals in cortical parenchyma was seen. Phloem was relatively narrow, made up of phloem parenchyma (occasionally, with some yellowish brown resin masses in inner cells) interlaid with scattered sieve tubes with companion cells and transverse by uni to biseriate ray cells. The secondary xylem region was separated from phloem by indistinct and narrow cambium represented almost two thirds the bulk of the root. Wood consisted of simple pitted wood parenchyma, numerous xylem vessels and fibres, tracheids and medullary rays. Vessels were radially arranged and found scattered in groups or singly nearer to central region with wide lumen (130-250 μ by 50-100 μ), tracheids 175-300 μ by 30-50 μ. Wood fibres were abundant and with simple pits. Medullary rays were uni to biseriate, oblong rectangular and 60-90 cells deep filled with abundant starch grains and occasionally found pitted.

## Powder microscopy

Powdered roots were light yellowish brown in colour with indistinct odour and mucilaginous test. Powder study showed the characteristic features listed below.

Fragments of isolated lignified cork cells were elongated, up to 90 μ in length. Xylem vessels were broadly cylindrical with bordered pitted and rarely with reticulated thickening, 130-250 μ in length and 50-100 μ in diameter. Tracheids pitted, with moderately thick, tapering, beaded walls, with relatively broad lumen and 175-300 μ in length and 30-60 μ in diameter. Xylem fibres were found to have thick heavily lignified walls showing small transverse pits on surface. Xylem parenchymatous cells had moderately thick walls and were found with frequently simple circular pits. Stone cells were scattered or in groups of six to ten cells, irregular in shape and highly thickened with narrow lumen (60-100 μ). Fragments of cortical parenchyma were filled abundantly with starch grains and oil globules. Medullary rays showed presence of starch grains. Starch grains were numerous, simple (4-6 μ) as well as 3 to 5 of them were compound (up to 20 μ), spheroid, ovate or irregular. Occasionally fragments containing mucilage were seen, which turned red when treated with ruthenium. Fragments of cortical and phloem parenchyma were filled with calcium oxalate crystals and resinous mass (Figure 3).

## Physicochemical parameters of roots of *G. arborea*

Powdered roots were evaluated for various physicochemical parameters and percentage of total ash, acid insoluble ash, water soluble ash were calculated (Table 1). As the drug is reported to contain various flavolignans, ethyl acetate soluble

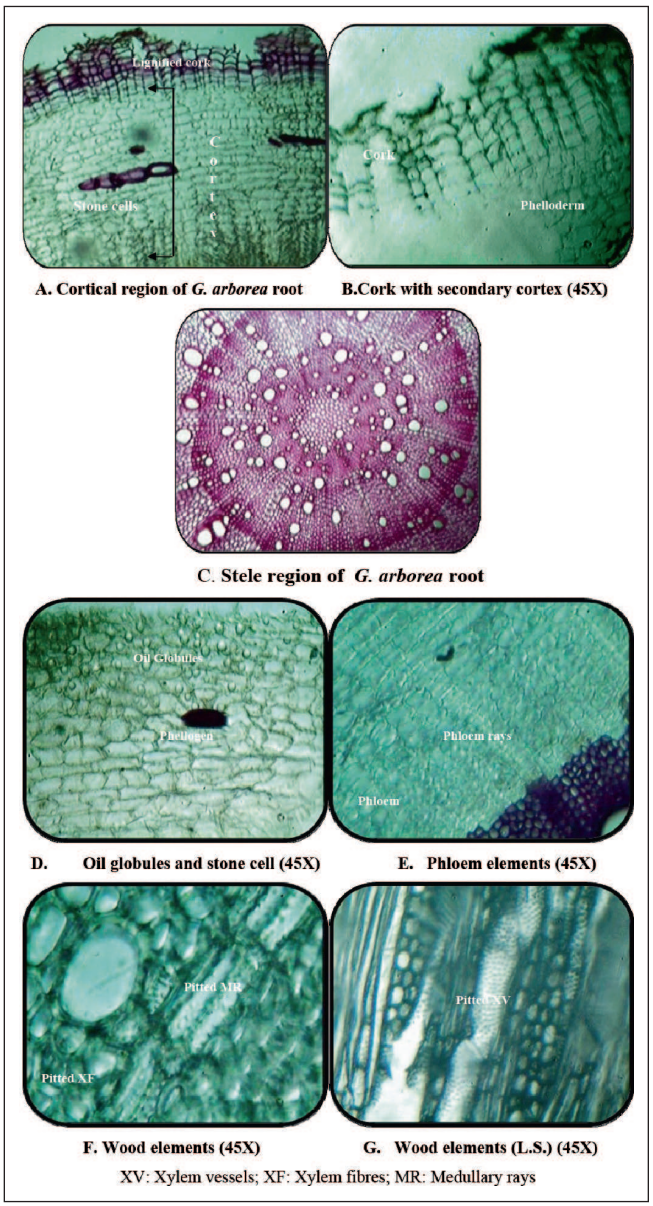


**Figure 2:** Microscopical characters of roots of *G. arborea*

extractive value was also determined for the roots along with the water soluble, alcohol soluble and ether soluble extractive values. The results of fluorescence analysis of the drug powder are presented in (Table 2).

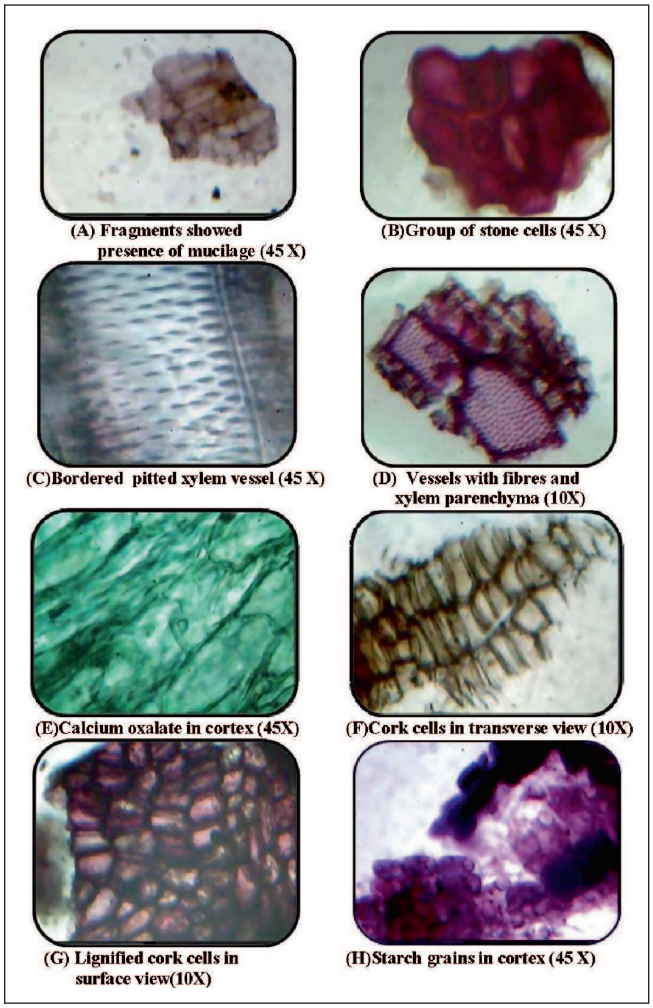


**Figure 3:** Powder study of roots of *G. arborea*

## Phytochemical screening

Preliminary phytochemical analysis showed the presence of various phytoconstituents through positive tests for flavonoids, lignans, sterols, tannins, carbohydrate, coumarins and alkaloids. It showed negative tests for saponins. Preliminary phytochemical screening revealed the presence of phenolics and tannins in methanolic and aqueous fraction and flavonoids and lignans in ethyl acetate fraction (Table 3). Presence of abundant phenolics and tannins in the preliminary screening led us to estimate the total phenolics content in the sample. The root powder was estimated for and found to contain 1.89% w/w phenolics.

## Estimation of β-sitosterol in roots of *G. arborea*

β-sitosterol along with other phytosterol have been reported in the roots and heartwood of this plant which was further confirmed in the present study by the phytochemical screening of the roots. HPTLC method was developed to quantify β-sitosterol in methanolic extract of the roots of *G. arborea*. β-sitosterol was estimated with very good resolution and the minimum interference with other constituents in mobile system toluene: methanol (9.4:0.6) and detected as a violet colored band with 0.5% anisaldehyde sulphuric acid reagent followed by heating at 110°C for

**Table 1: Physicochemical parameters of the roots of *G. arborea***

| Sr. No. | Parameters | % w/w |
|---|---|---|
| 1. | Total ash value | 1.712 |
| 2. | Acid insoluble ash | 0.29 |
| 3. | Water soluble ash | 0.03 |
| 4. | Moisture content | 67.8 |
| 5. | Water soluble extractive | 20.76 |
| 6. | Alcohol soluble extractive | 10.89 |
| 7. | Ethyl acetate soluble extractive | 1.86 |
| 8. | Petrol ether soluble extractive | 0.15 |

**Table 2: Fluorescence characteristics of the powdered roots of *G. arborea* with different reagents**

| Treatment | Ordinary light | UV light | |
|---|---|---|---|
| | | 254 nm | 365 nm |
| Powder as such | Yellowish brown | No color | No color |
| In 1N NaOH | Yellowish | Dark brown | Light brown |
| In 1N HCl | Yellow brown | Dark brown | Brown black |
| In 1N $HNO_3$ | Slightly red | Brown | Brown |
| In Conc. $H_2SO_4$ | Dark reddish brown | Black | Black |
| In 5% $FeCl_3$ | Brown yellow | Dark brown | Brown |
| In Iodine | Brown black | Black | Black |
| In Ammonia | Creamiest yellow | Greenish brown | Brown |
| In Acetic acid | Pale brown | Black | Black |
| In Picric acid | Yellow | Black | Black |
| In Conc. HCl | Dark brown | Black | Black |
| In Ammonia + $HNO_3$ | Dark yellow | Dark brown | Pale brown |
| In Methanol | Cream yellow | Dark green | Green black |
| In Ethanol | Yellow brown | Green | Green |
| In Distilled water | Yellow | No color | Yellow |

10 minutes at $R_f$ 0.31 ± 0.03 (Figures 4 and 5). It was then scanned at its absorption maxima 523 nm after derivatization. Four other bands resolved at different $R_f$ 0.12, 0.22, 0.43 and 0.7 were found to be resolved in methanolic extract in the developed mobile system. The identity was confirmed by co-chromatography and overlain absorption spectra with reference standard, when scanned at 523 nm (Figure 6). The method was precise and found to be linear in the range of 500-7500 ng/spot with the correlation coefficient of 0.992 (Figure 7). Limit of detection and limit of quantification

**Table 3: Preliminary phytochemical screening of the roots of *G. arborea***

| Sr. No. | Tests for phytoconstituents | Petroleum ether | Chloroform | Ethyl acetate | Methanol | Water |
|---|---|---|---|---|---|---|
| 1. | Alkaloids | -- | + | -- | + | -- |
| 2. | Flavonoids | -- | -- | ++ | ++ | + |
| 3. | Lignans | -- | -- | ++ | ++ | -- |
| 4. | Saponins | -- | -- | -- | -- | -- |
| 5. | Sterols | ++ | ++ | + | -- | -- |
| 6. | Carbohydrates | -- | -- | + | ++ | ++ |
| 7. | Phenolics and tannins | -- | -- | ++ | +++ | +++ |
| 8. | Coumarins | + | + | -- | -- | -- |
| 9. | Fats and fixed oils | + | + | + | -- | -- |

+ Less, ++ Moderate, +++ High, -- Negative

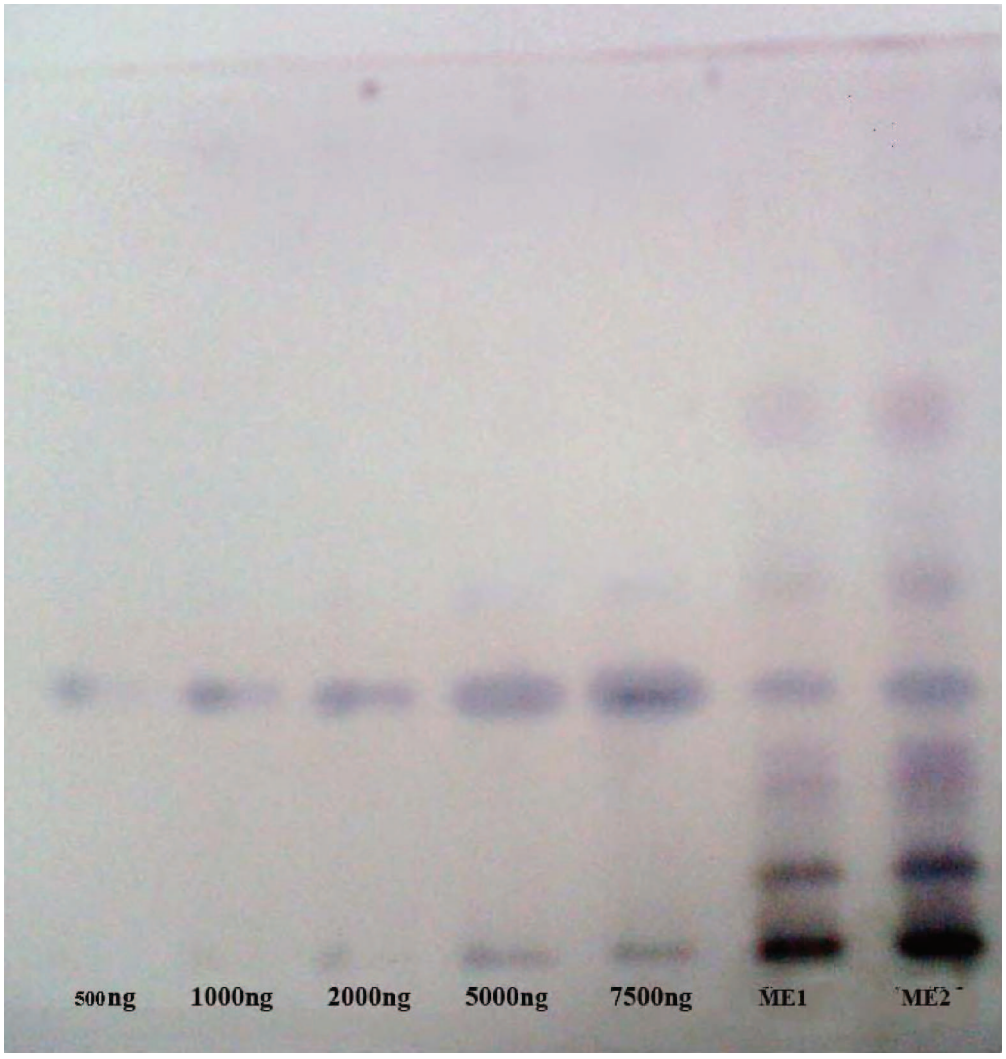


**Figure 4:** Densitometric chromatogram of β-sitosterol with methanolic extract of roots of *G. arborea* after derivatization with 0.5% anisaldehyde sulphuric acid reagent followed by heating at 110 °C for 10 minutes

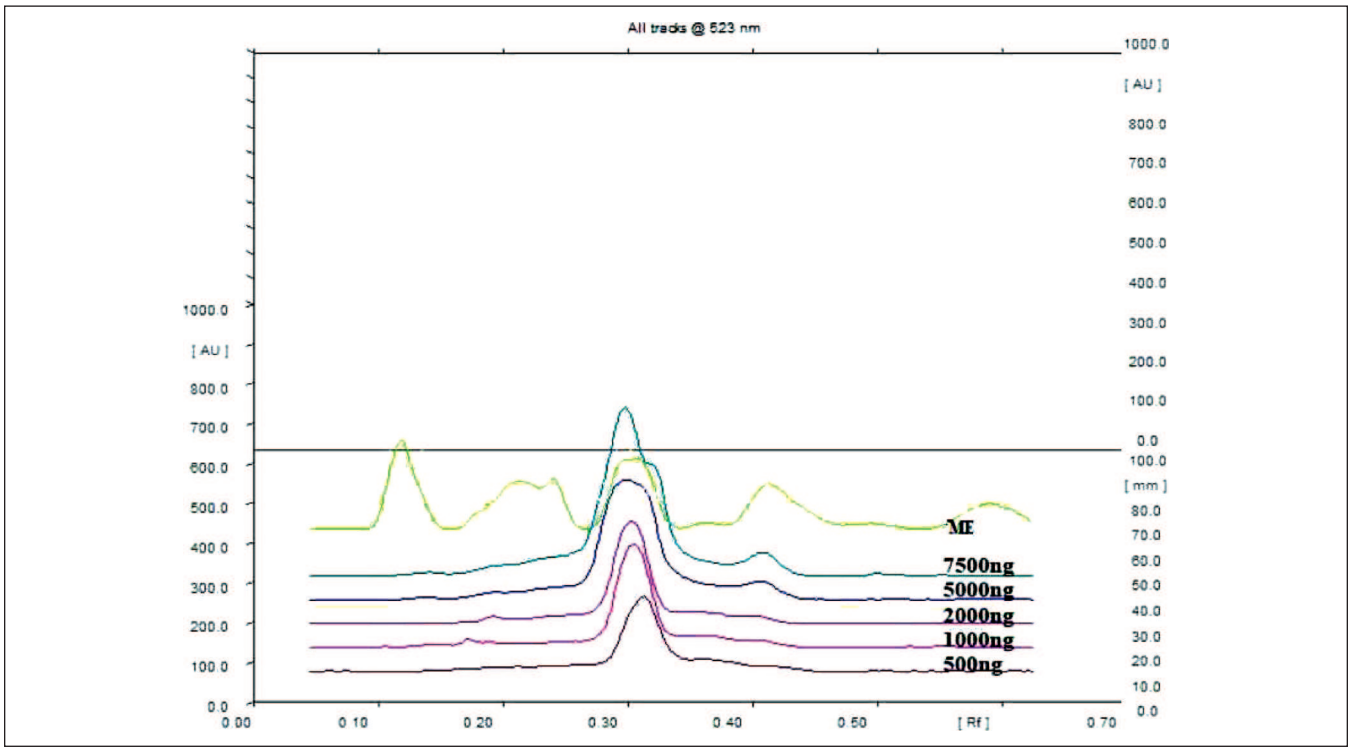


**Figure 5:** Densitometric chromatogram of β-sitosterol with methanolic extract of roots of *G. arborea* scanned at 523 nm

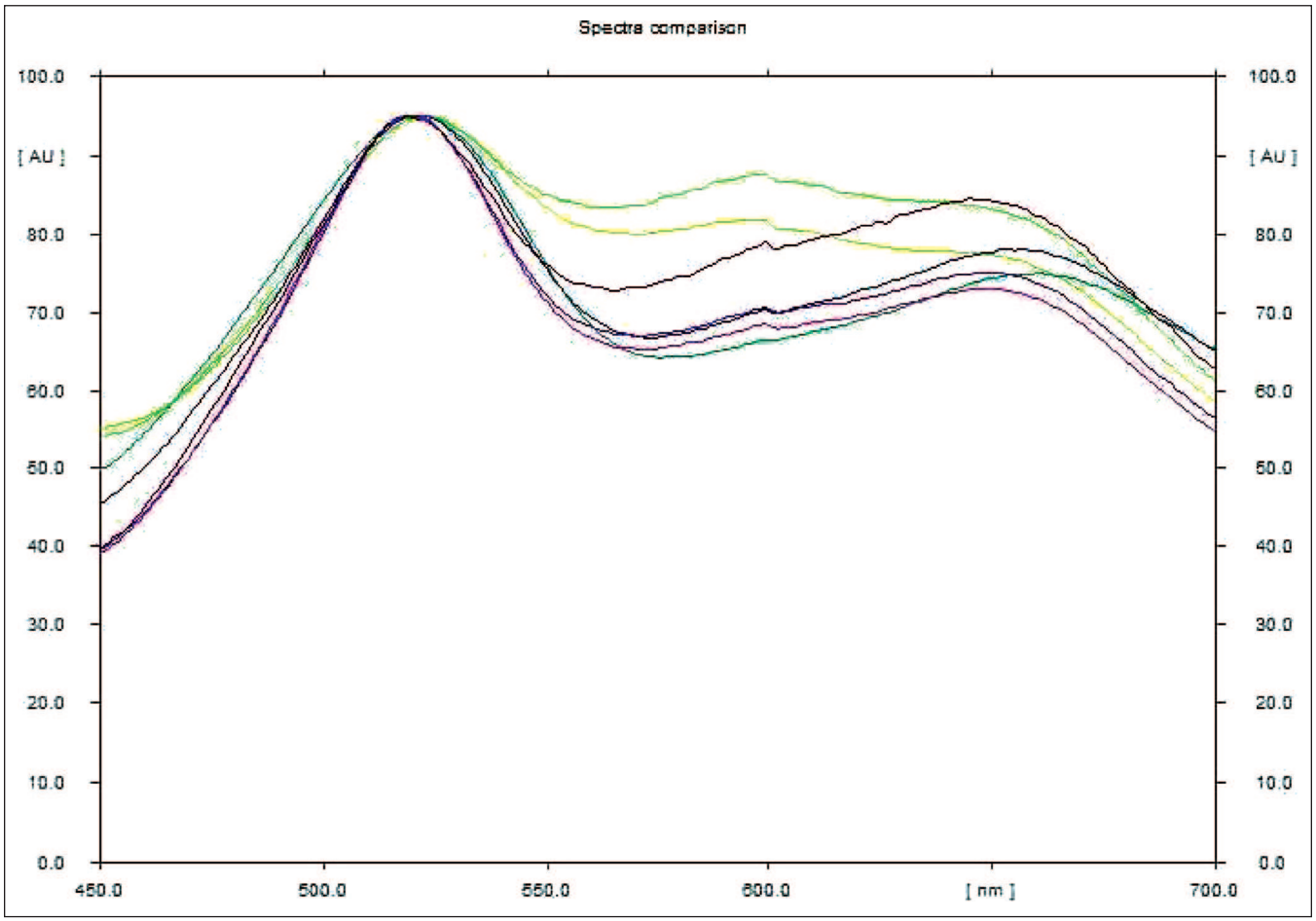


**Figure 6:** Overlain UV Spectrum of β-sitosterol with methanolic extract of roots of *G. arborea*

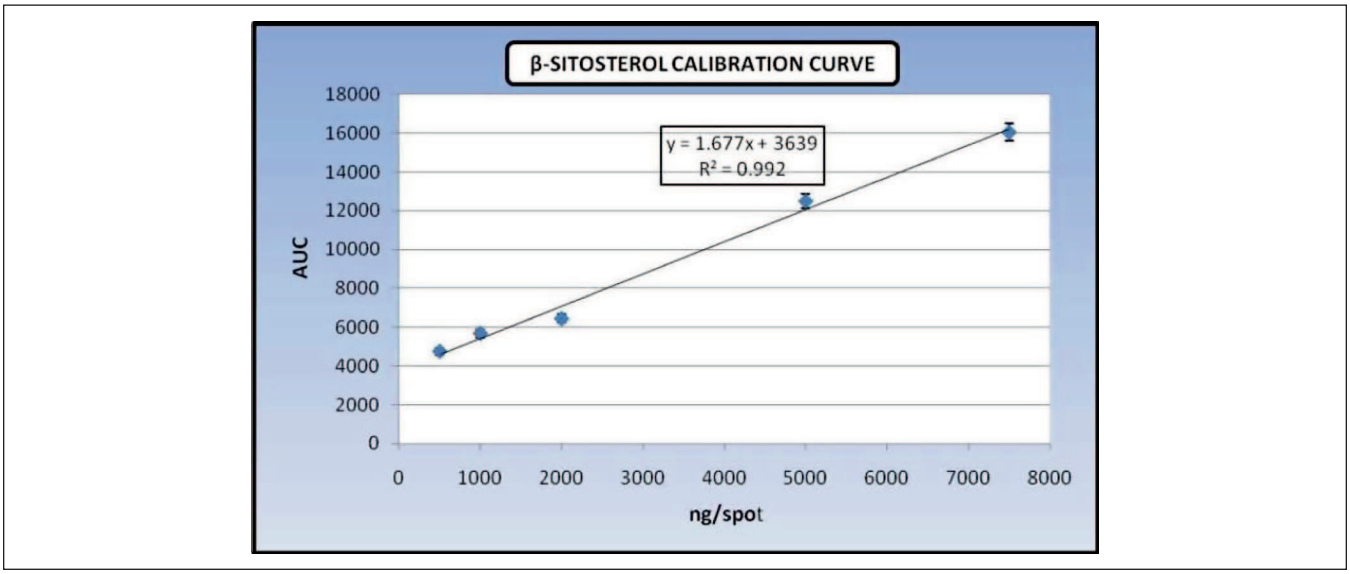


**Figure 7:** Calibration curve of standard β-sitosterol

**Table 4: Summary of validation parameters of β-sitosterol for the developed HPTLC method**

| Sr. No. | Parameter | Results |
|---|---|---|
| 1. | Linearity | 0.992 |
| 2. | Precision | |
| | • Repeatability of measurement | 3.76% |
| | • Repeatability of application | 3.65% |
| | • Intraday | 2.47-4.21% |
| | • Interday | 2.83-3.91% |
| 3. | Range of linearity | 500-7500 ng/spot |
| 4. | Limit of quantification | 500 ng |
| 5. | Limit of detection | 100 ng |
| 6. | Accuracy | 94.85-98.66% |
| 7. | Specificity | Specific |

was found to be 100 ng/spot and 500 ng/spot, respectively. The percentage content of β-sitosterol was found to be 0.120 ± 0.018 in the methanolic extract of the roots of this plant.

Validation of HPTLC method for estimation of β-sitosterol in roots of G. arborea

The correlation coefficient was found to be 0.992 with RSD, 2.83-3.91% in the developed method. The intraday and interday coefficient of variation for β-sitosterol varied from 2.83-3.91%, and 2.47-4.21%, respectively. RSD for repeatability of measurement of peak area based on 7 times measurement of 250 ng/spot of standard β-sitosterol was found to be 3.76%. RSD for repeatability of measurement of peak area based on a 7 time measurement of 1 µl/spot of sample extract was found to be 3.65%.

Percentage recovery of β-sitosterol was found to be in the range of 94.85-98.66, which indicated that the developed method was accurate and satisfactory, even though it was a derivatization method. The minimum detectable limit was found to be 100 ng/spot. Summary of all validation parameters for the developed HPTLC method for β-sitosterol are listed in Table 4.

## DISCUSSION

The improvement in the quality control and standardization of herbal drugs has led to the development of effective quality medicines from plants. In the present study, roots were evaluated qualitatively as well as quantitatively by studying various physicochemical parameters, phytochemical screening and by estimating for the presence of secondary metabolites. Detailed pharmacognostical studies for bark and heartwood has been reported for this drug but roots have not been studied for major microscopic and physicochemical properties. Further, root forms the major ingredient in Dashmoola and other preparations, so the present study is useful in establishing a monograph detail for the roots of *G. arborea*. Root powder was evaluated for its ash values, extractive values and loss on drying. In the present study, detailed physicochemical parameters and method for estimation of phenolics were developed and determined for the first time for the roots of *G. arborea* and to our knowledge this has not been reported earlier. Hence this analysis aids to set up certain standards and contribute towards the development of quality parameters for this drug.

The new HPTLC method developed for β-sitosterol was validated for specificity, linearity, accuracy, and precision. Accuracy was validated by analysis of spiked blank and standard addition samples and precision by performing

replicate analyses on a single day and on different days. It is apparent from the results that validation data for a developed quantitative HPTLC method for analysis of β-sitosterol meet the acceptance criteria for accuracy, precision, linearity and for detection and quantification limits. Thus, the developed HPTLC method can be used for routine analysis of β-sitosterol as a chemical marker compound in crude drugs as well as in herbal formulations containing *G. arborea*.

## CONCLUSION

Standardization of crude drugs has become very important for identification and authentication of a drug of natural origin. The present study was developed to establish the pharmacognosy, preliminary phytochemical analysis with other various physicochemical standards for the development of a monograph detail of highly valuable drug ingredient of Dashmoola preparation of Ayurveda. Development of detailed pharmacognostical parameters will be helpful in the correct identification of the plant specimen and authentification of the roots. Further, this is the first report for the estimation of total phenolics in roots and development of a validated HPTLC method for the characterization of β-sitosterol in the roots of *G. arborea*. Roots are widely used in many herbal and Ayurvedic formulations where the developed method can be used for the standardization of a chemo marker to ascertain the quality of the formulation.

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# Phytochemical Screening, DNA Fingerprinting, and Nutritional Value of *Plectranthus amboinicus* (Lour.) Spreng

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## ABSTRACT

In this study, *Plectranthus amboinicus* (Lour.) Spreng was subjected to phytochemical and genetic investigation, so that it could be properly identified. The results of phytochemical screening of the different plant organs (leaves, stems and roots) revealed the presence of steam volatile substances, sterols and/or triterpenes, flavonoids, carbohydrates and/or glycosides, catechol tannins in the three organs under investigation. A DNA sample of the plant was analyzed by Random Amplified Polymorphic DNA (RAPD) technique using eleven oligonucleotide primers. The analysis of RAPD data achieves the use of B-19 and B-6 primers for selective discrimination of this plant. Also, the nutritional value, including the total carbohydrates, total soluble sugars, proteins, amino acids and vitamin content, of the leaves, stems and roots was determined. The roots recorded the highest values of total carbohydrates, total soluble sugars and proteins content (66.04, 23.33 and 17.58 g%, respectively), followed by the leaves (48.12, 4.74 and 16.45 g%, respectively) and the stems (44.62, 3.10 and 9.52 g%, respectively). The three organs under investigation contain essential amino acids in moderate amounts compared to the WHO daily recommended doses. They are rich in vitamins and can be used as a vitamins supplement.

**Key words:** *Plectranthus amboinicus*, DNA fingerprinting, phytochemical screening, nutritional value

## INTRODUCTION

Genus *Plectranthus* belongs to family Lamiaceae and comprises about 350 species cultivated as ornamental plants or as sources of essential oils.[1] *Plectranthus amboinicus* (Lour.) Spreng (synonyms include *Plectranthus aromaticus* Roxb., *Coleus aromaticus* Benth. and *Coleus amboinicus* Lour.), is a perennial herb, native to Indonesia and is distributed in Tropical Africa, Asia and Australia. It is used as food, additive and fodder, and as medicine in treating a wide range of diseases.[1] The leaves extract is used to treat inflammatory disease,[2,3] chronic cough and urinary disease[2]. It is also used as an aromatic carminative and anthelmintic.[2] It was reported to have antimicrobial,[2,4] cytotoxic[2,3] and antioxidant activities[2,5]. Essential oils, diterpenes, flavonoids and phenolic acids are the main constituents isolated from the different *Plectranthus* species.[6,7] Because of taxonomic similarities of the different *Plectranthus* species, the same species of *Plectranthus* usually shows a number of synonyms.[1] To help in solving this terminology problem, in this study, the different organs (leaves, stems and roots) of *Plectranthus amboinicus* (Lour.) Spreng growing in Egypt were subjected to a phytochemical screening aiming for chemical identification of this plant and the DNA profile of the plant was analyzed for genetic identification. The total carbohydrates, total soluble sugars, proteins, amino acids and vitamin content were determined to evaluate the nutritional value of this plant as a food.

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## MATERIALS AND METHODS

### Plant material

*Plectranthus amboinicus* (Lour.) Spreng were collected over the years (2008-2010) from El-Orman garden. The plant was identified by Dr. Mohamed el Gebaly and Madam Treze (Taxonomist). A voucher specimen was kept in the herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

### Preliminary Phytochemical Screening

The powdered air-dried leaves, stems and roots of *Plectranthus amboinicus* (Lour.) Spreng were tested for the presence of steam volatile substances,[8] sterols and/or triterpenes,[9,10] flavonoids,[11,12] crystalline sublimate,[13] carbohydrates and/or glycosides,[14,15] tannins,[16] saponins,[17] alkaloids and/or nitrogenous bases,[18] anthraquinones,[19] cardiac glycosides,[8,20-23] and oxidase enzyme[8]. The results are given in table 1.

### DNA fingerprinting

DNA fingerprinting was performed in Agricultural Genetic Engineering Research Institute using the Random Amplified Polymorphic DNA (RAPD) technique.[24] DNA was extracted using CTAB extraction buffer (1% N-cetyl-N,N,N trimethyl ammonium bromide). Eleven oligonucleotide primers (Operon Technologies Inc., Almeda, California, USA) were used for analysis. Amplification of DNA was carried out in thermal cycler, programmed as follows: first cycle at 94 ºC for 5 min. (initial strand separation), followed by one cycle for 40 seconds at 94 ºC (denaturation), 1 min. at 36 ºC (annealing), forty cycles each for 1 min at 72 ºC (elongation) and the last cycle for 7 min at 72 ºC (final extension).

PCR reactions were performed in polypropylene tubes containing 2.5 µl reaction buffer, 2 µl $MgCl_2$, 2.5 µl of each dNTPs (Pharmacia, Sweden), 3µl primers, 0.5 µl Taq DNA polymerase (Perkin-Elmer/Cetus, USA; advanced Biotechnologies, UK), 3 µl template DNA, and enough sterilized water to obtain 25 µl.

PCR material was separated by horizontal electrophoresis in a 1.5% agarose gel plate (Sigma Co.). 10 µl of each PCR product was mixed with 3 µl loading buffer and loaded onto wells of the gels. The gels were run at 95 volts.

After electrophoresis the RAPD pattern was visualized by staining the gel with ethidium bromide solution (0.5 µg/ml), visualized under UV light, and photographed using a gel documentation system. RAPD molecular weight markers (Biolab Co.) were used. The banding profile produced by the eleven decamer primers is given in table 2.

**Table 1: Results of phytochemical screening of the different organs of *Plectranthus amboinicus* (Lour.) Spreng**

| Constituent | Leaves | Stems | Roots |
|---|---|---|---|
| Steam volatile substances | ++ | + | ± |
| Sterols and/ or triterpenes | ++ | + | + |
| Free aglycones | ++ | + | + |
| Flavonoids | + | + | + |
| Crystalline sublimate | - | - | - |
| Carbohydrates and/or glycosides | + | + | + |
| Catechol tannins | + | + | ++ |
| Pyrogallol tannins | - | - | - |
| Saponins | - | - | - |
| Alkaloids and/or nitrogenous bases | - | - | - |
| Free anthraquinones | - | - | - |
| Combined anthraquinones | - | - | - |
| Cardiac glycosides | - | - | - |
| Oxidase enzyme | - | - | - |

+: Present, ++: Strongly positive, -: Absent, ± : Traces

### Determination of total carbohydrates and total soluble sugars

The total carbohydrate and total soluble sugars were determined in the leaves, stems and roots by colorimetric method according to Duboies et al, (1956).[25]

### Determination of protein content

The total protein content was determined, in the three organs under investigation adopting Micro-Kjeldahl method according to A.O.A.C. (1995).[26]

### Determination of amino acids

The amino acids content in the three organs was determined by spectrophotometric method using amino acids analyzer (AAA 400, INGOS Ltd) after acid hydrolysis.[27] The results obtained are shown in table 3.

### HPLC analysis of vitamins

HPLC analysis of vitamins[28-31] was carried out on Agilent 1100 apparatus equipped with Hypersil-BDS-$C_{18}$ column (4.6 × 250 mm). The injection volume was 5 µl, the mobile phase was methanol at a flow rate of 1 ml/min. Detection was carried out with UV detector. The results are shown in table 4.

## RESULTS AND DISCUSSION

### Preliminary Phytochemical Screening

The results of phytochemical screening showed the presence of steam volatile substances, sterols and/or triterpenes, flavonoids, carbohydrates and/or glycosides, catechol tannins in all organs of *Plectranthus amboinicus* (Lour.) Spreng. Crystalline sublimate, saponins, alkaloids and/or nitrogenous bases, cardiac glycosides and oxidase enzyme are absent in all organs.

### Genetic profiling

The RAPD electrophoretic profile of the DNA sample amplified with the eleven decamer primers generated 58 fragment patterns, produced by B-19 (9 bands ), B-6 (9 bands), D-20 (8 bands), E-4 (6 bands), A-6 and G-5 (5 bands each), A-17 and G-2 (4 bands each), A-18 and G-17 (3 bands each) and G-19 (2 bands). Thus, primers B-19 and B-6 could be selected for discrimination of *Plectranthus amboinicus* (Lour.) Spreng.

**Table 2: Molecular size in base pairs of amplified DNA fragments produced by eleven decamer primers in *Plectranthus amboinicus* (Lour.) Spreng**

| Approx. band size (bp) | Decamer Primers | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | A6 | A17 | A18 | B6 | B19 | E4 | G2 | G5 | G17 | G19 | D20 |
| 1375 | | | | | + | | | | | | |
| 1343 | | | | + | | | | | | | |
| 1311 | + | + | | | | | | | | | |
| 1295 | | | | | | | | + | | | |
| 1280 | | | | | + | + | | | | | |
| 1265 | | | | + | | | | | | | |
| 1205 | | | | + | | | | | | | |
| 1191 | | | | | + | | | | | | |
| 1122 | + | | | | | | | | | | |
| 1108 | | | | | | | + | | | | |
| 1095 | | | | | | | | | | | + |
| 1069 | | | | | + | | | | | | |
| 1032 | + | | | + | | | | | | | |
| 1019 | | | | | | + | | | | | |
| 995 | | | | | | | | + | | | + |
| 983 | | | | | | + | + | | | | |
| 960 | | | | | + | | | | + | | |
| 949 | | | | | | | + | | | | |
| 842 | | | | | | + | | | | | |
| 822 | | | | | | | | | | | + |
| 812 | | | | + | | | | | | | |
| 793 | | | | | + | | | | | | |
| 774 | | + | | | | + | | | | | |
| 756 | | | | | | | | + | | | |
| 747 | | | | | | | + | | | | |
| 720 | | + | | + | | | | | | | + |
| 695 | | | | | | + | | | | | |
| 670 | | + | | | | | | | + | | |
| 631 | | | | | | | | + | | + | |
| 609 | | | | | | | | | | | + |
| 602 | | | + | | | | | | | | |
| 595 | + | | | | + | | | | | | |
| 574 | | | + | | | | | | | | |
| 553 | | | | | + | | | | + | + | + |
| 534 | | | | + | | | | | | | |
| 521 | | | | | | | | + | | | |
| 503 | | | | | | | | | | | + |
| 479 | | | | + | | | | | | | |
| 468 | | | | | | | | | | | + |
| 451 | | | + | | | | | | | | |
| 441 | | | | | + | | | | | | |
| 391 | | | | + | | | | | | | |
| 343 | + | | | | | | | | | | |
| Sum | **5** | **4** | **3** | **9** | **9** | **6** | **4** | **5** | **3** | **2** | **8** |

+: Present

## Nutritional value

Total carbohydrates were found to be 48.12, 44.62 and 66.04 g% of dry weight in the leaves, stems and roots, respectively. The total soluble sugars recorded 4.74, 3.10 and 23.33 g% of dry weight in the leaves, stems and roots, respectively. The protein content of the leaves, stems and roots was 16.45, 9.52 and 17.58 g% of dry weight, respectively. Aspartic acid (25.02, 25.80 and 19.35 mg/100 g of dry weight), glutamic acid (12.71, 10.79, and 13.23 mg/100 g of dry weight) and glycine (12.45, 12.77 and 14.02 mg/100 g of dry weight) were the main identified amino acids in the leaves, stems and roots, respectively. Meanwhile, the major detected amino acids were non-essential amino acids, the three organs under investigation contained essential amino acids (threonine, valine, isoleucine, leucine, phenyl alanine, histidine and lysine) in moderate amounts compared to the WHO daily recommended doses.[32] The three organs are rich in vitamins

**Table 3: Amino acids in *Plectranthus amboinicus* Lour. (Spreng) leaves, stems and roots**

| Amino acid | Percentage of amino acid (mg/100 g of dry weight) | | |
|---|---|---|---|
| | Leaves | Stems | Roots |
| Aspartic acid | 25.02 | 25.80 | 19.35 |
| Thrionine | 4.16 | 5.33 | 4.49 |
| Serine | 5.66 | 6.76 | 6.77 |
| Glutamic acid | 12.71 | 10.79 | 13.23 |
| Proline | 0.09 | 0.10 | 0.04 |
| Glycine | 12.45 | 12.77 | 14.02 |
| Alanine | 8.48 | 10.73 | 9.92 |
| Valine | 5.82 | 0.55 | 7.30 |
| Isoleucine | 2.61 | 2.93 | 4.17 |
| Leucine | 7.15 | 8.66 | 7.86 |
| Tyrosine | 1.04 | 0.69 | 0.84 |
| Phenyl alanine | 3.81 | 3.83 | 3.58 |
| Histidine | 2.09 | 2.35 | 4.27 |
| Lysine | 4.45 | 5.81 | 3.43 |
| Arginine | 4.43 | 2.85 | 0.71 |

**Table 4: Vitamins content in the leaves, stems and roots of *Plectranthus amboinicus* Lour. (Spreng)**

| Vitamins | Leaves | Stems | Roots |
|---|---|---|---|
| Vitamin A (ppm) | 0.38 | 0.12 | 0.02 |
| Vitamin E (ppm) | 2.93 | 1.26 | 0.33 |
| Vitamin D (ppm) | 4.471 | 0.0313 | 0.209 |
| Vitamin C (%) | 0.11 | 0.11 | 0.12 |
| Vitamin B complex (%) | | | |
| Thiamin B1 | 0.03 | 0.01 | 0.02 |
| Pyridoxin B6 | 0.01 | -- | 0.008 |
| Riboflavin B2 | 2.47 | 0.91 | 0.7 |
| Nicotinic B3 (Niacin) | 0.52 | 0.08 | 0.1 |
| Cyanocobalamine B12 | 0.37 | 0.14 | -- |
| Folic acid | 0.002 | -- | 0.03 |

according to the WHO daily recommended doses,[33] and can be used as a vitamins supplement. From the results obtained, it could be concluded that *Plectranthus amboinicus* (Lour.) Spreng is a useful edible plant, can be used as a herbal nutrition supplement and can be considered as a multivitamins supplementary agent, for vitamins deficiency patients, or individuals on a restricted diet. This may explain its traditional use as a food or food additive.

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# Pharmacognostical Evaluation, Phytochemical Analysis and Antioxidant Activity of the Roots of *Achillea tenuifolia* LAM.

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## ABSTRACT

**Introduction:** *Achillea* L. (Asteraceae) contains about 100 species throughout the world with many therapeutic aspects. *Achillea tenuifolia* is one of the mentioned species that grows wildly in Iran. **Methods:** In this research the antioxidant activity of methanol and ethyl acetate extracts obtained from the roots of *A. tenuifolia* against free DPPH (2,2-diphenyl-1-picrylhydrazyl) radical together with the total phenol contents of extracts were assayed. Furthermore, preliminary phytochemical analysis of the above mentioned extracts and microscopic characterization of various plant tissues were determined. **Results:** The results showed that total phenol contents of methanol and ethyl acetate extracts were 59.4 ± 1 and 70.6 ± 3.8 (GAE µg/mg EXT), respectively. $IC_{50}$ value for BHA, vitamin E, methanol and ethyl acetate in radical inhibition were calculated in the following order: 7.8, 14.2, 145.5 and 320 µg/mL. The scavenging capacity of methanol extract was higher than ethyl acetate extract. Preliminary phytochemical analysis indicated that both extracts contained sterols and terpenoids, nevertheless, tannins were detected in the methanol extract. Microscopic observations exhibited the presence of undeveloped cypsela, papillae stigma and elongated epidermal cells in the flower tissue, lanceolate leaflet with anomocytic stomata, cubic calcium oxalate prism and oil containing cells in the leaf parts, anomocytic stomata and cicatrix in the stem segments and finally sclereids, pitted and spiral vessels in the root tissue. **Conclusion:** Root extracts of *A. tenuifolia* mainly contain tannins, terpenes and sterols, and shows antioxidant activity not necessarily related to their total phenol content. Different plant tissues exhibited characteristic microscopic properties which make it distinguishable from other *Achillea* species.

**Key words:** *Achillea tenuifolia*, antioxidant activity, microscopy, preliminary phytochemical analysis

## INTRODUCTION

Microscopy characterization of medicinal plants is a valuable procedure for identity and quality assessment of herbal ingredients. It is well accepted by all national and international regulatory authorities as one of the four primary methodologies for the identification of crude drug materials including macroscopic appearance, organoleptic characters, microscopic characteristics, and the presence or absence of chemical substances.[1,2] Identification of some specimens is very difficult, this is especially true for *Achillea* species which has different varieties, including wild and ornamentals.[1] The genus *Achillea* is well represented in Flora Iranica with about 100 species, 19 of them grow in Iran.[3] *A. tenuifolia* LAM. (Asteraceae) is distributed in the north and north-west of Iran. It is a perennial herbaceous plant, woody at base, with many stems and growing up to 25-90 cm.[4] Phytochemical investigations of *Achillea* species have exhibited bioactive components such as flavonoids, phenolic acids, coumarins, terpenoids, sterols, alkamides and volatile oils.[5-7] Many species of this genus have been used as traditional herbal remedies against fever, common cold, hemorrhage, pneumonia, rheumatic pain and digestive complaints. These are topically used for healing wounds and skin inflammation. In addition recent studies confirmed pharmacological and biological activities of this genus such as antioxidant, anti-tumor, anti-spasmodic, estrogenic, anti-spermatogenic, and treatment of alimentary tract disease.[6-8]

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Literature review revealed that there is no report about the microscopy analysis of this species. Only chemical and physical characteristics of *A. tenuifolia* seed oil have been determined by gas chromatography which contains linoleic (69.4%) and oleic (14.5%) acids as the most abundant fatty acids.[9] The antioxidant activity, total phenols and total flavonoids of the aerial parts of *A. tenuifolia,*[10] together with the cytotoxicity evaluation of the mentioned extracts against the larvae of *Artemia salina*[11] have been previously reported.

In the present study methanol and ethyl acetate extracts of *A. tenuifolia* have been reported for antioxidant activity, total phenol content assay, preliminary phytochemical investigation, and microscopic evaluation of various parts of the plant.

## MATERIALS AND METHODS

### Plant Material

All parts (leaves, stems, flowers and roots) of *A. tenuifolia* were collected from Qazvin province (1500 m) in June 2011, and identified by Mr. Yousef Ajani. A herbarium specimen (No. 1604) has been deposited at the Herbarium of Institute of Medicinal Plants, Jahade-Daneshgahi (ACECR), Karaj, Iran. The plant materials were cleaned and dried in shade at room temperature. Each part of the plant was separated and crushed for microscopic investigation.

### Extraction

The powdered plant material was extracted (700 g) by maceration method in ethyl acetate and methanol, consequently, three times for each solvent at room temperature. The extracts were concentrated after removing the solvent by rotary evaporator and then lyophilized using a freeze dryer. The concentrated methanol and ethyl acetate extracts weighed as 2.7 and 1.91 g (on the basis of dry weight), respectively. The extracts were then kept in opaque containers under cold and dry conditions until assay.

### Free Radical Scavenging

Free radical scavenging activity of the root extracts has been evaluated by 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) modified method.[12] Free stable radical DPPH has been widely used to elucidate the free-radical scavenging of natural antioxidants. One mL of different concentrations of the extracts (100-500 µg/mL) was added to 2 mL of DPPH ($4 \times 10^{-2}$ mg/mL in methanol). The absorptions at 517 nm were measured after 30 min. Free radical 50% inhibition ($IC_{50}$) provided by extracts concentrations were determined from the plot of inhibition percentage against extract concentration. The assay was carried out in triplicate. Vitamin E and BHA were used as positive standards.

$$\% \text{ Oxidation} = \left[\left(\frac{\text{Abs. sample} - \text{Abs. control}}{\text{Abs. blank}}\right) \times 100\right]$$

$$\% \text{ Inhibition} = 100 - \% \text{ Oxidation}$$

### Total Phenol Assay

Total phenolic contents were examined as GAE, expressed as µg GAE $mg^{-1}$ extract.[13] Different concentrations of the root extracts (1 mL) were transferred to glass tubes, to which 5 mL Folin-Ciocalteu reagent (diluted 1:10) was subsequently added and incubated at room temperature for 10 min. Four milliliter of sodium bicarbonate (75 mg/mL) was added to the mixture and it was made up to 10 mL with distilled water. Each solution was incubated for 30 min at room temperature, and then its absorbance was measured at 765 nm. The sample absorbance was compared to gallic acid absorption. All determinations were carried out in triplicate and the mean values were presented (Figure 1).

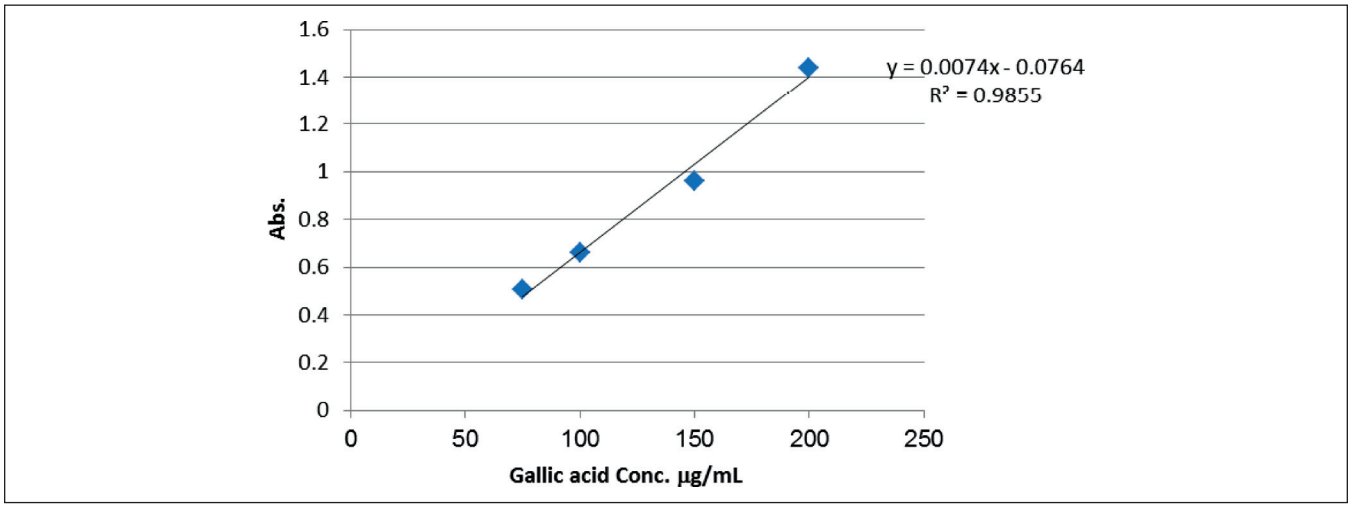


**Figure 1:** Gallic acid standard curve obtained from total phenol assay.

### Statistical Analysis

Comparisons between controls and extract antioxidation activity have been done in triplicate sets. The data were recorded as mean ± standard error and analyzed by SPSS (Version 11.5, SPSS Inc.). *P* values < 0.05 were regarded as significant.

### Preliminary Phytochemical Analysis

In order to determine the various classes of natural compounds in the ethyl acetate and methanol extract of *A. tenuifolia,* preliminary screening tests for detection of alkaloids, flavonoids, sterols, tannins and terpenoids were carried out on the basis of those reported in the literature.[14]

### Microscopic Observations

One gram of each tissue powder (leave, flower, stem and root) of *A. tenuifolia* was separately boiled in potassium hydroxide solution (10%) in a backer on heater for 30 seconds (or 1 minute) depending on the tissue hardness, and washed afterwards with distilled water three times. The powders were successively treated with sodium hypochlorite for bleaching and then washed with distilled water. The preparation was mounted in aqueous glycerin.[15] Photomicrographs were taken using Zeiss microscope attached with a digital camera. Photomicrographs of sections were taken at different magnifications depending upon the microscopic details to be observed.

## RESULTS

### Radical Scavenging and Total Phenol Contents

Values of $IC_{50}$ for radical scavenging in methanol and ethyl acetate extracts were calculated as 145.5 and 320 µg/mL, respectively. $IC_{50}$ in free radical inhibition for standard vitamin E (14.2 µg/ mL) and BHA (7.8 µg/ mL) were also measured (Figure 2). Total phenol contents were measured as 59.4 ± 1 and 70.6 ± 3.8 (GAE µg/ mg EXT) for the methanol and ethyl acetate extracts, respectively. The extent of DPPH inhibition of the methanol extract (100 µg/mL) showed the same activity as 10 µg/mL of vitamin E. The DPPH inhibition of ethyl acetate extract in 300 and 400 µg/mL were similar and they significantly displayed lower radical scavenging activity as compared to 500 µg/mL of this extract. However, the methanol extract indicated higher activity in comparison to ethyl acetate extract of the roots. DPPH inhibition for both of methanol (300 µg/mL)

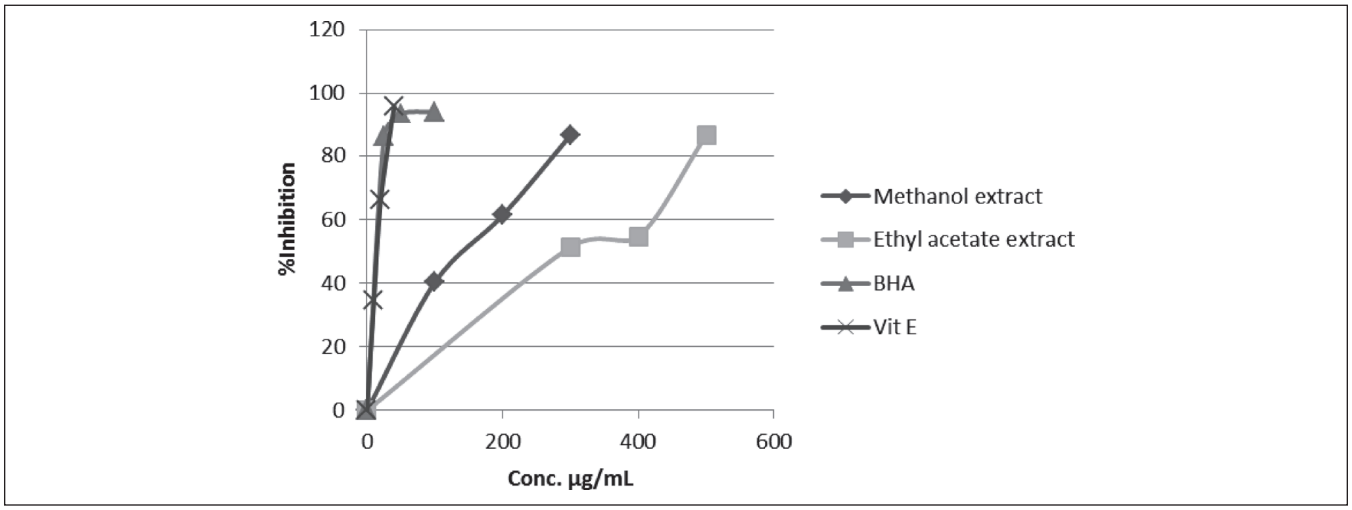


**Figure 2:** Evaluation of methanol and ethyl acetate extracts of *A. tenuifolia* compared to the standards BHA and Vitamin E, obtained from DPPH inhibitory assay.

**Table 1: Qualitative phytochemical analysis of *A. tenuifolia* root methanol and ethyl acetate extracts**

| Test | Reagent | Observation | Methanol extracts | Ethyl acetate extracts |
|---|---|---|---|---|
| Alkaloids | Meyers and Wagner reagent | Cream and orange precipitate | Negative | Negative |
| Flavonoids | HCl plus Amylic alcohol | Reddish color | Negative | Negative |
| Tannins | $FeCl_3$10% | Blue color | Positive | Negative |
| Sterols | Sulfuric acid (conc.) | Reddish brown interface | Positive | Positive |
| Terpenoids | Glacial acetic acid plus Sulfuric acid (conc.) | Reddish brown interface | Positive | Positive |

and ethyl acetate (500 μg/mL) extracts were observed the same as 25 μg/mL of BHA (positive standard).

### Preliminary Phytochemical Analysis

Phytochemical screening of both extracts revealed the presence of sterols and terpenoids. Tannin was also present in the methanol extract of the root (Table 1).

### Microscopic Observations

Microscopic characterization of the plant flower was assessed, epidermis with oblong cells, undeveloped cypsela, papillae stigma and part of the style (Figure 3). In the leaf sample, the leaflet exhibited a lanceolate tip and consisted of the oil-containing cells, together with the cubic calcium oxalate prism (Figure 4). Upper epidermis made up of elongated cells and lower epidermis composed of slightly elongated cells with sinuous walls, but both epidermis of the leaf consisted anomocytic stomata (Figure 4). Stem epidermis demonstrated cicatrix and anomocytic stomata the same as the flower sample with abundant covering trichomes (Figure 5). Sclereids, pitted and spiral vessels were observed in the roots (Figure 6).

## DISCUSSION

Although, the total phenol contents of ethyl acetate extract were higher than methanol extract, the latter showed 50% inhibition of DPPH in a lower concentration and was a better free-radical scavenger. Antioxidant activity of an extract can be the result of various active components such as peptides,

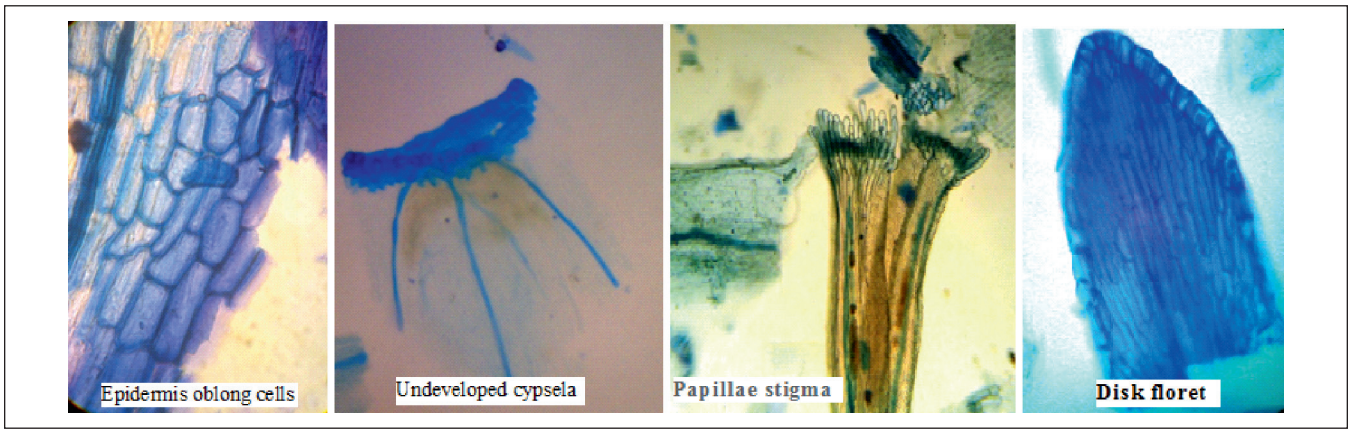


**Figure 3:** Microscopic characterization of *A. tenuifolia* flower.

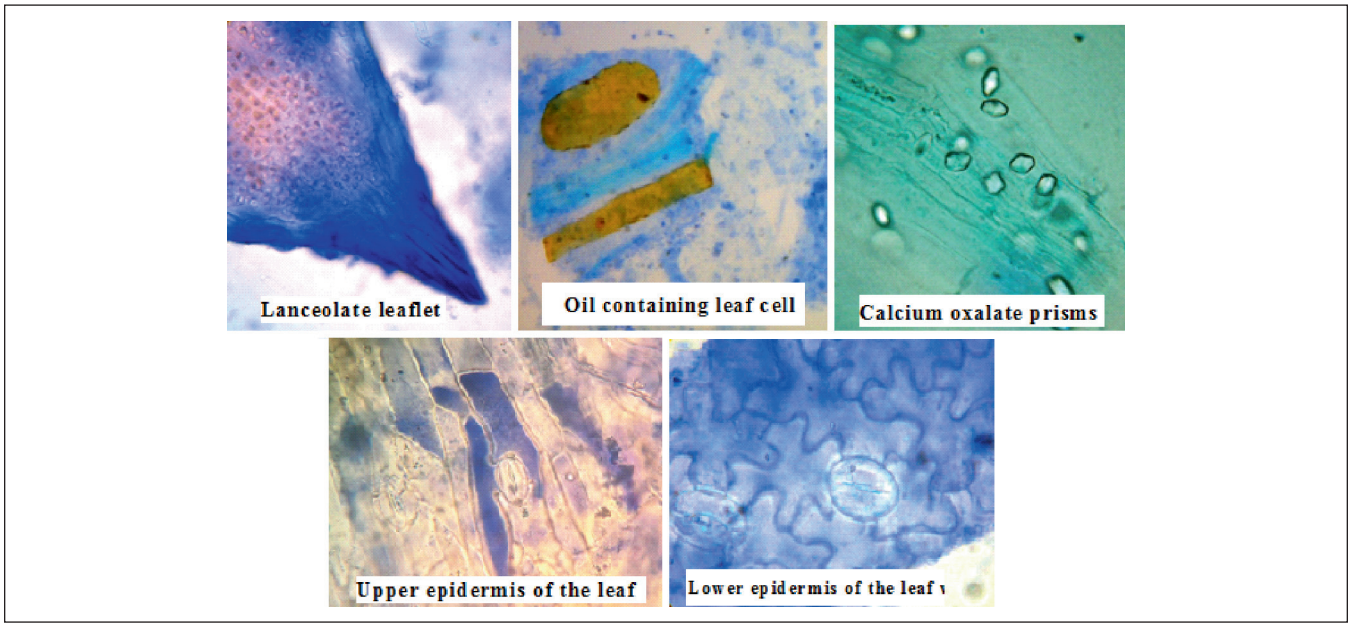


**Figure 4:** Microscopic characterization of *A. tenuifolia* leaf.

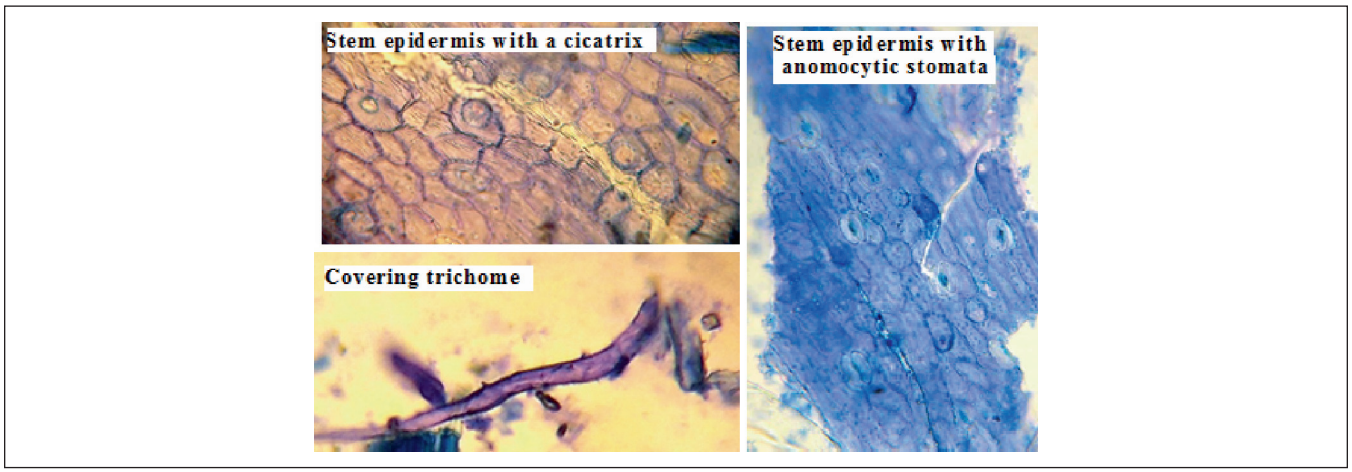


**Figure 5:** Microscopic characterization of *A. tenuifolia* stem.

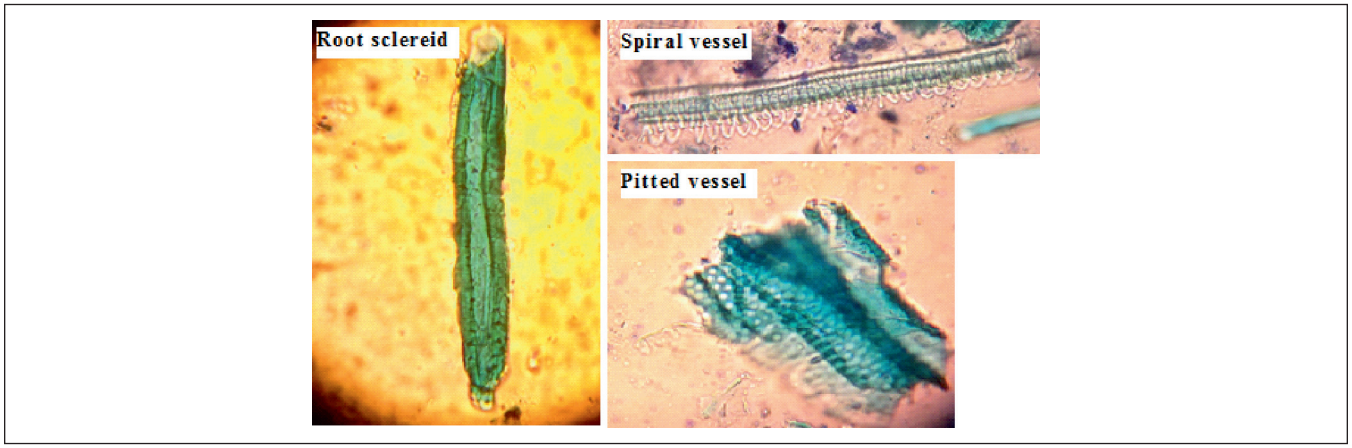


**Figure 6:** Microscopic characterization of *A. tenuifolia* root.

organic acids, phenolics and so on.[16] In the previous report, total phenol contents of the aerial parts of *A. tenuifolia* were calculated as 43.97 ± 0.034, 74.16 ± 0.55 and 106 ± 0.693 mg/g in the ethyl acetate, methanol and aqueous-methanol extracts, respectively. Methanol and aqueous-methanol extracts exhibited comparable scavenging activity to the BHT, which can be due to the phenols and flavonoids contents.[10] In the present study, ethyl acetate extract of the roots showed higher amount of phenolics than methanol extract. This makes the roots considerable for more phytochemical analysis.

Among the several species of *Achillea, A. millefolium* is the most popular medicinal plant used for various medicinal properties and introduced as the officinal species in different pharmacopoeias. For this reason, microscopic identification of *A. millefolium* is noteworthy to distinguish any adulterants or other similar species especially in crude herbal mixtures. Microscopic investigation of this study revealed some similar characteristics in the flower samples of *A. tenuifolia* and *A. millefolium*. Undeveloped cypsela and papillae stigma have been found in both flowers. *A. millefoilum* shows coriaceous bristle-like tip of a leaflet but *A. tenuifolia* leaflet is in lanceolate shape. Anomocytic stomata were illustrated in both species, although upper epidermis of *A. tenuifolia* has elongated leaf cells without wavy cell walls.[1] Stem sample of *A. tenuifolia* displays anomocytic stomata, cicatrix and covering trichome with elongated cell. Additionally, sclereids and vessels tissue can be observed in powdered plant roots.

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# Antidiabetic Activity of Standardized Extracts of *Balanites aegyptiaca* Fruits using Cell-based Bioassays

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## ABSTRACT

The antidiabetic activities of different extracts and fractions of *Balanites aegyptiaca* were tested in cultured C2C12 skeletal muscle cells and 3T3-L1 adipocytes. An 18-h treatment with 200 µg/mL of the sugars fraction (**A1**) showed the highest activity as it increased basal glucose uptake by 52% in muscle cells; which is twice the activity of 100 nM insulin [insulin equivalent (IE) = 2.0 ± 0.07]. The dichloromethane (**E**) and ethyl acetate (**F**) successive extracts exerted 37 and 41% increase in the glucose uptake, respectively. Only **E** and **F** accelerated the triglyceride accumulation in pre-adipocytes undergoing differentiation, comparably with 10 µM rosiglitazone [rosiglitazone equivalent (RE) was 1.6 ± 0.3 and 0.7 ± 0.1, respectively). Gas chromatography (GC) analysis of **A1** revealed the presence of xylose, rhamnose, sorbitol, fructose, galactose and glucose. The active extracts **E** and **F** were standardized by high-performance liquid chromatography (HPLC) to contain 0.031 and 0.239% of rutin, 0.007 and 0.004% of isorhamnetin, respectively.

**Key words:** balanites, C2C12, diabetes, fruit, sugars, 3T3-L1.

## INTRODUCTION

*Balanites aegyptiaca* Del. (Zygophyllaceae) is used traditionally in African countries as an anthelmintic[1] and in the treatment of jaundice.[2] The fruits are used as an oral antihyperglycemic in Egyptian folk medicine[3] and herbalists in the Egyptian market sell the fruits as an antidiabetic agent. However, quality control of such herbal products remains a great challenge. The aqueous extract of the mesocarp of the Balanites fruits revealed significant antidiabetic activities in STZ-induced diabetic rats and mice[3,4] and several saponins were isolated from the mesocarp.[3,5-8] Nevertheless, the target tissues and mechanisms of action of this herb are not yet well understood.

The aim of the present study was to assess the antihyperglycemic activities of different extracts and fractions of *Balanites aegyptiaca* fruits defining target tissues and the corresponding group of bioactive compounds through cell-based bioassays using C2C12 myotubes and 3T3-L1 adipocytes.

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## MATERIALS AND METHODS

### Plant material

The fruits of *Balanites aegyptiaca* were brought from Aswan and were authenticated by Prof. Dr. M. Gebali (Plant Taxonomy and Egyptian Flora Department, National Research Center, Giza, Egypt). A voucher specimen (voucher no. 201) was deposited at the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

### Extraction and fractionation

Balanites fruits (1 kg) were defatted by *n*-hexane and successively extracted by maceration in each of dichloromethane, ethyl acetate, methanol and water. Extracts were filtered and concentrated under vacuum at 50 °C giving extracts **E**, **F**, **G** and **H**, respectively. Similarly, the fruit was extracted with methanol, cold water, hot water and 70% ethanol to give extracts **A**, **B**, **C** and **D**, respectively. The dried methanol extract (3 gm) was defatted with n-hexane. The mother liquor was chromatographed on Diaion HP-20 (Supelco, St. Louis, MO, USA) column and eluted with $H_2O$, 40% methanol and 95% methanol successively, giving 31, 12.5 and 19% of free sugars and other aqueous soluble substances (**A1**), as well as substances that do not dissolve completely in water, like phenolics (**A2)**

and saponin-rich (**A3**) fractions, respectively.[9,10] The dried extracts and fractions were dissolved in dimethyl sulfoxide (DMSO; 0.1% final concentration) for biological screening.

### HPLC analysis of active extracts

An HPLC method was adapted for analysing *Balanites aegyptiaca* **E** and **F** extracts. An Agilent 1100 series HPLC was used, equipped with Agilent G1311A quaternary pump, a G1314A variable wavelength detector and a G1328A manual injector. *Balanites aegyptiaca* ethyl acetate and dichloromethane fractions were dissolved in methanol (17.24 and 22.34 mg/ml, respectively), filtered through PTFE 0.45 µM syringe filter (Macherey-Nagel, Germany) and injected into a Hypersil 100 RP-18, 5 µm, 250 × 4 mm column. The mobile phase used was acetonitrile (solvent A) and 1% acetic acid in water (solvent B). Gradient elution was carried out at a flow rate of 1.0 ml/min as follows: 0-20 min 30 to 75% A in B. Measurements were made with an injection volume of 20 µl and UV detection at 370 nm. Standard calibration curves were prepared using five concentrations of isorhamnetin (1, 2, 4, 8 and 10 µg/ml) and rutin (4, 8, 10, 16 and 20 µg/ml) in methanol. For each sample, three replicate assays were performed.

### GC analysis of the sugar fraction

Fraction **A1** (70 mg) was extracted with hot water and derivatised.[11] The derivatised sugar solution (1 ul) was injected onto a 3 m × 250 µm capillary column packed with 14% cyanopropyl phenyl methyl (HP-1701) heated isothermally at 150 °C for 2 min, then temperature programmed at 7 °C/min to 200 °C. GC apparatus HP6890 was used and injector/detector (FID) temperature was 270 °C with nitrogen carrier gas at 40 ml/min. Sugar standards (xylose, rhamnose, sorbitol, fructose, galactose, glucose and glucuronic acid) were similarly prepared and injected to identify the retention time of each.

### Cell culture

C2C12 myoblasts and 3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were cultured as previously described in Dulbecco's modified Eagle's medium.[12-14] C2C12 cells were treated with 200 µg/mL of extracts and fractions. Concentrations of 50 or 100 µg/mL were used for treating the 3T3-L1 preadipocytes. Diosgenin 50 µM (Sigma-Aldrich, Germany) was used for treating both cell lines. These concentrations were based on the maximum non-toxic concentrations determined in the employed cell lines following 18-h treatment.

### Glucose transport assay

Differentiated C2C12 cells grown in 12-well culture plates were treated with the test solutions, vehicle control (0.1% DMSO) and a positive control (400 µg/mL metformin) for 18 h. Insulin 100 nM was added to one well as a second positive control during the assay.[12,14,15] Five separate experiments, each with three replicates, were performed.

### Adipogenesis assay

3T3-L1 cells seeded in 12-well plates were treated with the test solutions or 0.1% DMSO vehicle in differentiation medium during the entire differentiation period as described before.[12,14,15] Rosiglitazone 10 µM (Alexis Biochemicals, San Diego, CA, USA), dissolved in DMSO, was used as a positive control. Three separate experiments, each with three replicates, were performed.

## RESULTS AND DISCUSSION

### HPLC finger-print chromatograms of active extracts

Successive extracts **E** and **F** were analyzed by HPLC to develop a fingerprint chromatogram for each. The extracts were injected against available flavonoid reference compounds (rutin, isorhamnetin, quercetin, scopoletin, caffeic acid and hyperoside). Rutin and isorhamnetin were identified and used for standardization of the extracts (Figure 1). The concentrations of rutin in *Balanites aegyptiaca* **F** and **E** extracts were found to be 0.239 and 0.031% and those of isorhamnetin were 0.004 and 0.007%, respectively.

### GC analysis of the sugar fraction

A GC chromatogram was developed as a fingerprint for the most active sugar fraction **A1** (Figure 2). The relative retention times for xylose, rhamnose, sorbitol, fructose, galactose, glucose and glucuronic acid were 7.30, 8.29, 9.66, 9.80, 10.76, 11.07 and 14.25 min, respectively. Xylose, rhamnose, sorbitol, fructose, galactose and glucose were detected in the sugar fraction with area percentages of 1.35, 26.37, 1.18, 1.51, 49.22 and 13.2%, respectively, with an unknown peak at retention time 8.18 min constituting 4.96% of the total areas. A previous analysis of the glycosyl part identified within balanites fruit revealed the presence of galactose, mannose, arabinose, xylose, rhamnose and glucuronic acid.[16]

### Cell-based bioassays for antidiabetic activity

The enhancement of glucose uptake in muscle cells and the acceleration of triglyceride accumulation in differentiating adipocytes (increased adipogenesis) were studied for the plant extracts and fractions. The four extracts of balanites (**A**, **B**, **C** and **D**) exhibited similar significant activities on differentiated C2C12 myotubes as shown by their activities relative to an optimal dose of insulin (100 nM; insulin equivalents, IE: values of 0.7 ± 0.03, 0.6 ± 0.02, 0.7 ± 0.02 and 0.8 ± 0.02, respectively) (Figure 3). Extraction of the defatted fruits with solvents of increasing polarity resulted in an increase in activity to become about 1.5-fold that of

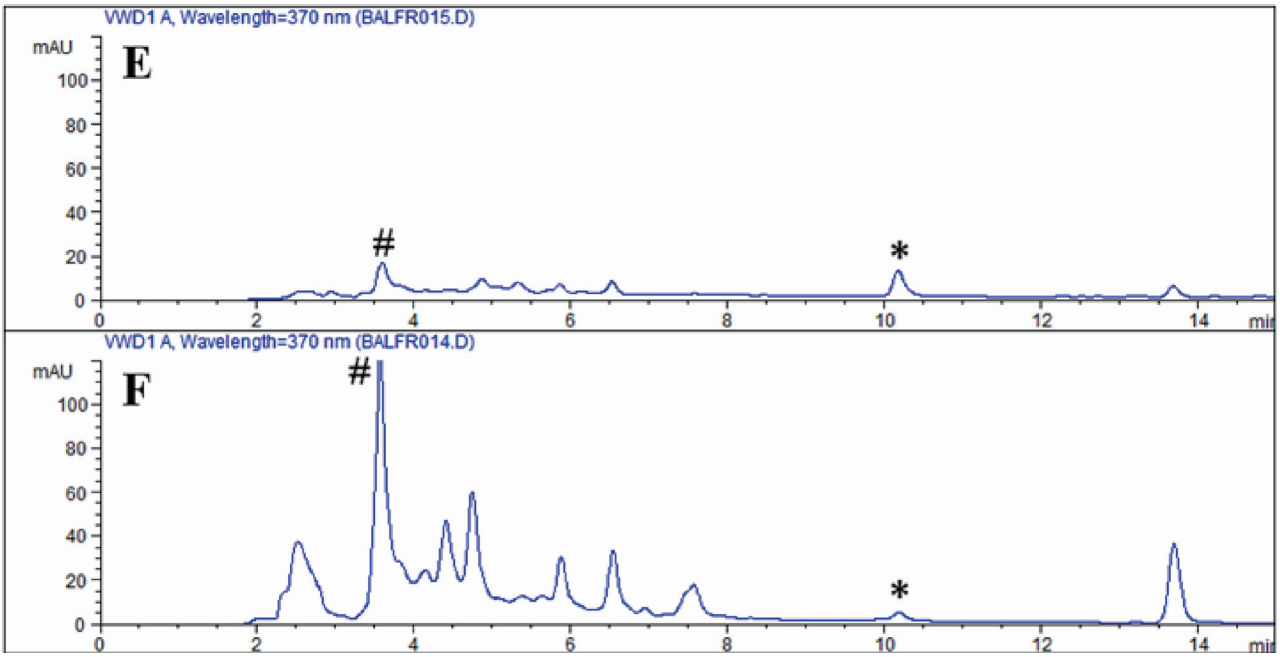


**Figure 1:** HPLC chromatograms of extracts **E** and **F** showing peaks of rutin (#) and isorhamnetin (*) at retention times (min) 3.5 and 10.2, respectively. Gradient elution was carried out using acetonitrile and 1.0% acetic acid in water. Peaks were detected at 370 nm.

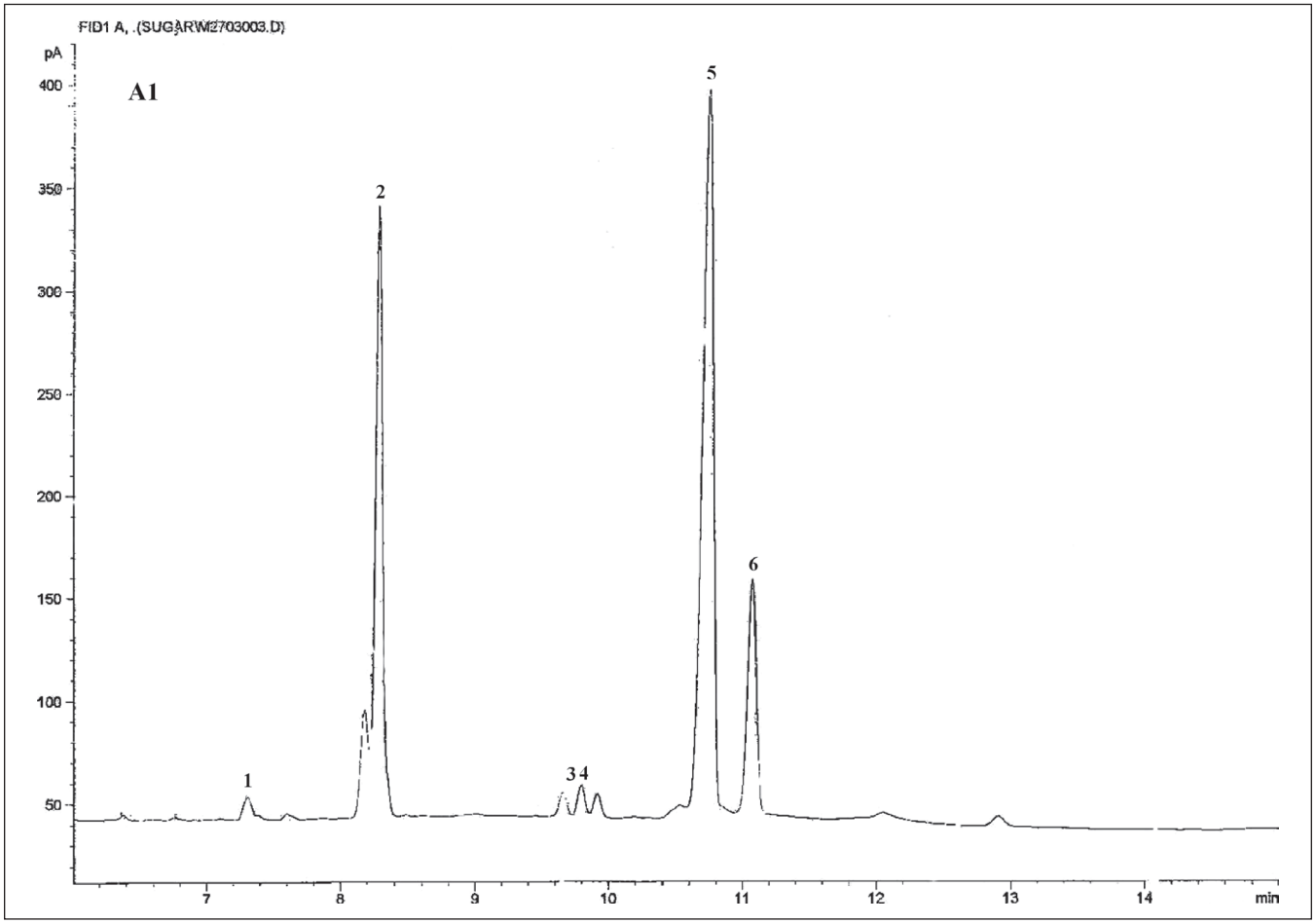


**Figure 2:** GC chromatogram of fraction **A1** showing peaks of xylose (**1**), rhamnose (**2**), sorbitol (**3**), fructose (**4**), galactose (**5**) and glucose (**6**) at retention times (min) 7.30, 8.29, 9.66, 9.80, 10.76 and 11.07, respectively.

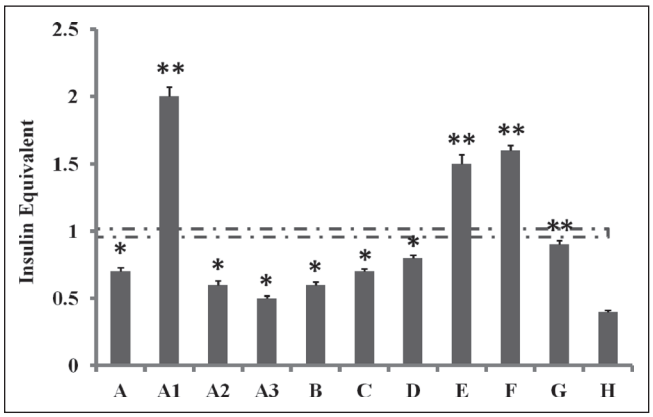


**Figure 3:** Basal $^{3}$H-deoxyglucose uptake in C2C12 myotubes treated with 200 µg/mL of each of A, A1, A2, A3, B, C, D, E, F, G and H or 0.1% DMSO (vehicle) for 18 h. Data was normalized to basal uptake in vehicle control condition and expressed as Insulin Equivalent (IE). Dotted lines represent the IE of 100 nM insulin (1 ± 0.02) that corresponds to 125.5 ± 3% $^{3}$H-deoxyglucose uptake (expressed as % of vehicle). Mean ± SEM for n = 3 to 5. The symbols (*) and (**) indicate a significant difference ($p \leq 0.05$ and $p \leq 0.0001$, respectively) from vehicle control.

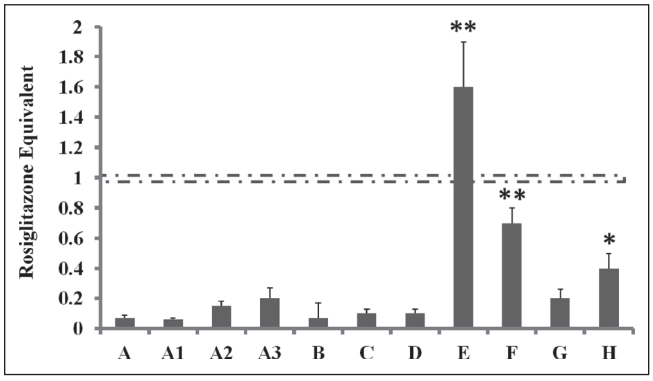


**Figure 4:** The rate of accumulation of triglycerides in differentiating 3T3 pre-adipocytes treated for 6 days with 50 µg/mL of each of A, A1, C, D, G and H and 100 µg/mL of each of A2, A3, B, E and F, 0.1% DMSO (vehicle) or 10 µM rosiglitazone in differentiation medium. Results were normalized to vehicle control in differentiation minus vehicle control in proliferation medium, and expressed as Rosiglitazone Equivalent (RE). Dotted lines represent the RE of 10 µM rosiglitazone (1 ± 0.07) that corresponds to 648 ± 51% intracellular triglyceride content (expressed as % of vehicle). Mean ± SEM for n = 3 to 4. The symbols (*) and (**) indicate a significant difference ($p \leq 0.05$ and $p \leq 0.0001$, respectively) from vehicle control.

insulin in successive extracts **E** and **F** (IEs of 1.5 ± 0.07 and 1.6 ± 0.04, respectively). Only these two extracts revealed a significant activity in increasing the triglyceride accumulation in differentiated 3T3-L1 adipocytes comparable to that caused by 10 µM rosiglitazone (RE = 1.6 ± 0.03 and 0.7 ± 0.1, respectively) (Figure 4). Both extracts were standardized to their phenolic content (Figure 1). Many studies have already demonstrated the antihyperglycemic activity of flavonoids using different experimental models.[14,17,18] Fractionation of extract **A** on Diaion HP-20 gave fraction **A1** standardized to contain monosaccharides and possessing the highest activity among all tested extracts and fractions. Indeed, it increased basal glucose uptake in skeletal muscle cells twice as much as insulin (IE = 2.0 ± 0.07) (Figures 2 and 3). Diosgenin 50 µM, the main aglycone of the saponins of Balanites, did not show any activity on the two cell lines under the experimental conditions (results are not shown). Kamel et al., (1991)[3] reported previously that oral administration of 80 mg/kg bw of the aqueous extract of the fruit mesocarp, as well as the polysaccharide fraction (precipitated by excess of alcohol) and the supernatant (saponin-rich), revealed significant antidiabetic activities in STZ-induced diabetic mice. In another study on STZ-diabetic rats, it was suggested that the antihyperglycemic effect of balanites aqueous extract (1.5 g/kg bw) was mediated through insulinomimetic effect as well as inhibition of intestinal α-amylase activity.[4]

The present studies bring forth novel data showing that the reported antihyperglycemic activity of *B. aegyptiaca* can also be attributed to significant insulin-like and partly glitazone-like activities in peripheral tissues. Increased muscle basal glucose uptake thus participates in the traditionally known, and *in vivo* proven, antidiabetic effect of the balanites fruits. Moreover, the sugar part (**A1**) and the phenolic content (**E** and **F**) contribute to a large extent to this activity. On the other hand, some of the balanites bioactive fractions (**E** and **F**) may act in a manner similar to the thiazolidinedione (e.g. rosiglitazone) family by increasing insulin sensitivity in the fat tissue.[19] Further investigations are in progress to isolate bioactive compounds from these active fractions, possessing a greater activity compared to insulin, which could hold a great promise for developing oral antihyperglycemic lead compounds.

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# Effects of Curcumin and Telmisartan on Olanzapine and high fructose diet induced Metabolic Syndrome in Sprague Dawly Rats

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## ABSTRACT

**Objective:** To study the effects of curcumin and telmisartan on olanzapine, high fructose diet (HFD) and both olanzapine and HFD-induced metabolic syndrome. **Method:** Adult male Sprague Dawly (SD) rats were used for the study. Animals were divided into ten groups including normal and HFD controls. The obesity/metabolic syndrome was induced using olanzapine (1 mg/kg; *i.p.*), a second generation antipsychotic and HFD. Effect of curcumin (50 mg/kg; *p.o.*) and telmisartan (5 mg/kg; *p.o.*) on olanzapine, HFD and both olanzapine and HFD-induced metabolic syndrome. All drugs were administered for 28 days. The blood sample was collected through retro orbital sinus and the plasma was separated at the end of study. The plasma sample was used to estimate the biochemical parameters such as blood glucose, aspartate aminotransferase (ALT), alanine aminotransferase (ALT), urea, uric acid, creatinine, total cholesterol, triglyceride; and high-density lipoprotein (HDL) levels were analyzed and the low-density lipoprotein (LDL) levels were calculated using Frieldwann's equation. After completing the experiment, all experimental animals were sacrificed by cervical dislocation and their organs such as heart, liver, kidney and peritoneal fat pads were removed and the absolute organ weight was measured. **Result:** The animals treated with olanzapine and fed with HFD showed significant increase in body weight and biochemical parameters when compared to the control group. Curcumin and telmisartan were significantly ($P$<0.001) inhibited, the HFD and olanzapine induced increase in body weight and also brought the biochemical levels to normal when compared to the olanzapine and HFD groups, respectively. The animals treated with telmisartan showed significant inhibition on the liver weight and peritoneal fat levels when compared to control group. **Conclusion:** The study results suggested that curcumin and telmisartan significantly inhibited the olanzapine and HFD-induced metabolic syndrome. Telmisartan affects the total lipid profile, glucose metabolism and decreases the body weight.

**Keywords:** curcumin, metabolic syndrome, telmisartan, olanzapine.

## INTRODUCTION

Antipsychotic agents are one of the most commonly used drugs to calm/sedate, control the symptoms of mania and relieve acute positive symptoms of schizophrenia, etc. in the 21st century. The most common adverse effect observed in antipsychotic drug therapy is rapid weight gain that is causing obesity and other metabolic syndromes. From past evidence we know that patients treated with 2nd generation antipsychotic drugs for a long term showed significant weight gain.[1,2]

Olanzapine is an atypical antipsychotic, approved by Food and Drug Administration (FDA) for the treatment of schizophrenia and bipolar disorder. It is structurally similar to clozapine, but is classified as a thienobenzodiazepine.[3] Long term administration of olanzapine is associated with high weight gain. Weight gain especially when manifested as intra-abdominal obesity, is a significant long term health issue as it is associated with insulin resistance and resultant metabolic effects such as elevated triglycerides, diabetes and hypertension, all of which increase cardiovascular

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diseases.[4,5] Olanzapine administration causes increase in ghrehlin which is responsible for increase in body weight.[6] Patients with schizophrenia often have immediate concerns regarding weight gain and poor physical function, discomfort in public places, poor self-esteem and problems with sexual performance. Increase in body weight may cause metabolic syndrome such as insulin resistance, hypoinsulinamia, glucose intolerance, adipogenesis, dyslipidemia and hypertension.[7,8] Like olanzapine, high fructose diet (HFD) also causes insulin resistance, hypertension, hepatic stress and weight gain.[9,10,11,12] Curcumin and telmisartan are a natural anti-oxidant and angiotensin II receptor antagonist, respectively. The anti-obesity and insulin resistant properties of this compound have already been proved.[13,14] Hence, the present study was planned to determine and compare the effect of curcumin and telmisartan on both olanzapine and HFD-induced metabolic syndrome.

## MATERIALS AND METHODS

### Animals

Experimental study was carried out using adult male Sprague Dawly (SD) rats weighing between 140-160 g. The animals were housed in clean, hygienic and large polypropylene cages. Animals were acclimatized to light and temperature with a 12h-12h dark-light cycle. The rats were fed with normal rodent pellet diet and water *ad libitum*. The rat pellets were supplied by M/s. Hindustan Lever Ltd., Bangalore, India. During the course of the experiments, the respective HFD-fed animal groups received HFD diet. The study protocol was approved by the Institute Animals Ethics Committee (IAEC/NCP/40/10), and all animal experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

### Metabolic syndrome inducing agents

High fructose diet (HFD) containing fructose 624 g/kg (62%), fats as vegetable oils 5 g/kg, protein 223 g/kg (22%), necessary amino acids, vitamins 1.25% and minerals.[15,16] Olanzapine (t.i.d) at a dose range of 0.5-2.0 m/kg mimics the clinical features in animals such as weight gain associated with hyperphagia, enhanced feeding efficiency, adiposity and altered locomotor activity and satiety signaling in 14 days.[10,17] In the present study, 1mg/kg olanzapine (o.i.d) was used for a duration of 28 days to induce obesity in SD rats.

### Effects of curcumin and telmisartan on olanzapine and HFD induced metabolic syndrome

Induction of insulin resistance in the experimental animals was carried out by feeding HFD. The animals were divided into the 10 groups, each comprising six animals as follows:

Group I : Control (fed with normal diet chows)
Group II : High fructose diet (HFD) control
Group III : Olanzapine (1 mg/kg)
Group IV : HFD + Olanzapine 1 mg/kg
Group V : HFD + Curcumin 50 mg/kg
Group VI : Olanzapine 1 mg/kg + Curcumin 50 mg/kg
Group VII : HFD + Olanzapine 1 mg/kg + Curcumin 50 mg/kg
Group VIII : HFD + telmisartan 5 mg/kg
Group IX : Olanzapine + Telmisartan 5 mg/kg
Group X : HFD + Olanzapine 1 mg/kg + Telmisartan 5 mg/kg

Curcumin (50 mg/kg b.wt) and telmisartan (5 mg/kg b.wt) doses were used for this study.[18,19] The standard drug and investigational produces were administered orally for 28 days. The metabolic syndrome was induced by treating/feeding the experimental animals with olanzapine or HFD. Olanzapine was administered during mornings and all other investigational products were administered during afternoon timings. During the study period, the weekly body weight variation was determined. The animals were fasted for 24h and the blood sample was collected through retro orbital sinus at the end of the study.[20] The blood samples were collected in sodium ethylenediaminetetraacetic acid (EDTA) tubes, centrifuged at 3000 RPM for 20 min and subjected to biochemical analysis (Vital Scientific N.V., The Netherlands). Plasma samples were used to estimate the biochemical parameters such as blood glucose, AST, ALT, urea, uric acid, creatinine, total cholesterol, triglyceride and HDL levels. The lipid profile, liver enzyme profile and glucose levels were estimated using Randox (E-merk India Ltd.) and Lab kit enzymatic kits, respectively. The LDL levels were calculated using Frieldwann's equation.[21,22] At the end of the study, all experimental animals were sacrificed by cervical dislocation[23] and organs such as heart, liver, kidney and peritoneal fat pads were removed and the absolute organ weight was measured and relative organ weight was calculated.

### Statistical Analysis

The mean and standard error of mean (SEM) values were calculated for each parameter. Significant differences between the groups were determined using repeated Analysis of Variance (ANOVA) measures followed by Dunnett's test. A $P$ value less than 0.05 was considered significant.

## RESULT

Effect of telmisartan and curcumin on body weight in HFD and olanzapine-induced metabolic dysfunction in rats is presented in Table 1. The HFD, olanzapine and both HFD

and olanzapine administered groups showed a significant ($P<0.001$) increase in body weight when compared to the control group at the end of the study. At the same time, curcumin and telmisartan significantly ($P<0.001$) inhibited HFD and olanzapine induced increase in body weight when compared to the HFD-fed animals and olanzapine administered group. Olanzapine, HFD + olanzapine and HFD + olanzapine + telmisartan treated groups significantly increased the relative organ weight of liver ($P<0.05$). Like HFD, olanzapine and HFD + olanzapine treated animals showed significant increase in the peritoneal fat levels when compared to the control ($P<0.01$). Telmisartan in HFD treated animals, showed significant inhibition on the liver weight. In telmisartan treated animals, marked changes in reduction in peritoneal fat levels were observed, but it was not statistically significant (when compared to control group).

Effect of curcumin and telmisartan on biochemical and lipid profiles is presented in Tables 2 and 3. HFD, olanzapine and HFD and olanzapine combined groups showed

**Table 1: Effect of curcumin and telmisartan on absolute body weight and relative organ weight in olanzapine and HFD induced insulin metabolic syndrome**

| Groups | Absolute body weight | Relative organ weight | | | |
|---|---|---|---|---|---|
| | | Heart | Liver | Kidney | Peritoneal fat |
| Group-I | 192.33 ± 1.73 | 0.32 ± 0.01 | 2.38 ± 0.08 | 0.67 ± 0.01 | 0.70 ± 0.02 |
| Group-II | 284.50 ± 3.18*** | 0.36 ± 0.01 | 2.42 ± 0.02 | 0.65 ± 0.01 | 1.46 ± 0.05** |
| Group-III | 234.17 ± 1.92*** | 0.34 ± 0.01 | 3.00 ± 0.15** | 0.67 ± 0.05 | 1.28 ± 0.05** |
| Group-IV | 305.50 ± 2.09*** | 0.36 ± 0.02 | 3.12 ± 0.11** | 0.65 ± 0.02 | 1.54 ± 0.06** |
| Group-V | 215.67 ± 3.84&&& | 0.31 ± 0.02 | 2.27 ± 0.06 | 0.66 ± 0.02 | 0.71 ± 0.06 |
| Group-VI | 192.83 ± 1.58### | 0.34 ± 0.02 | 2.39 ± 0.06 | 0.68 ± 0.04 | 0.67 ± 0.10 |
| Group-VII | 197.67 ± 2.14$$$ | 0.38 ± 0.02 | 2.41 ± 0.05 | 0.61 ± 0.03 | 0.97 ± 0.09 |
| Group-VIII | 183.33 ± 2.36&&& | 0.31 ± 0.01 | 1.79 ± 0.06** | 0.69 ± 0.02 | 0.62 ± 0.14 |
| Group-IX | 179.17 ± 1.49### | 0.36 ± 0.02 | 2.11 ± 0.07 | 0.65 ± 0.01 | 0.62 ± 0.05 |
| Group-X | 172.83 ± 1.82$$$ | 0.37 ± 0.01 | 2.12 ± 0.05 | 0.66 ± 0.02 | 0.62 ± 0.04 |

Values are mean ± SEM (N=6). *$P$<0.05; **$P$<0.01; ***$P$<0.001 as compare to control; &&&$P$<0.001 as compare to HFD; ###$P$<0.001 as compare to olanzapine; $$$$P$<0.001 as compare to olanzapine + HFD. One way ANOVA followed by Dunnett's multiple comparison tests.

**Table 2: Effect of curcumin and telmisartan on lipid profile in olanzapine and HFD-induced insulin resistance and metabolic syndrome**

| Groups | Total cholesterol | Triglycerides | HDL Levels | LDL levels |
|---|---|---|---|---|
| Group-I | 56.167 ± 1.662 | 74 ± 1.983 | 33.3 ± 0.766 | 25.667 ± 1.174 |
| Group-II | 74.833. ± 1.493*** | 143.333 ± 2.108*** | 24.333 ± 1.054*** | 31.162 ± 1.078** |
| Group-III | 65.667 ± 1.282** | 111.833 ± 2.400*** | 20.66 7 ± 1.145*** | 31.667 ± 1.606*** |
| Group-IV | 85.167 ± 1.537*** | 171.667 ± 3.169*** | 15.333 ± 0.989*** | 36 ± 0.775*** |
| Group-V | 46.833 ± 3.106&&& | 82.833 ± 1.60&&& | 34.5 ± 0.99&&& | 21.333 ± 0.80&&& |
| Group-VI | 47.667 ± 0.84### | 64.167 ± 1.76### | 34.5 ± 0.96### | 20.167 ± 0.70### |
| Group-VII | 68.167 ± 1.17$$$ | 103.833 ± 1.33$$$ | 24.333 ± 1.12$$$ | 21.667 ± 0.99$$$ |
| Group-VIII | 46.667 ± 1.783&&& | 64.833 ± 1.33&&& | 35.5 ± 0.67&&& | 20.333 ± 0.62&&& |
| Group-IX | 43.5 ± 0.89### | 64.667 ± 1.54### | 36.833 ± 0.83### | 18 ± 1.06### |
| Group-X | 63.167 ± 1.40$$$ | 88 ± 2.08$$$ | 35 ± 0.52$$$ | 20.5 ± 0.43$$$ |

Values are mean ± SEM (N=6). **$P$<0.01, ***$P$<0.001 as compare to control; &&&$P$<0.001 as compare to HFD; ###$P$<0.001 as compare to olanzapine; $$$$P$<0.001 as compare to olanzapine + HFD. One way ANOVA followed by Dunnett's multiple comparison tests.

**Table 3: Effect of curcumin and telmisartan on biochemical parameters in olanzapine and HFD induced insulin resistance and metabolic syndrome**

| Groups | Blood glucose | AST | ALT | Urea | Uric acid | Creatinine |
|---|---|---|---|---|---|---|
| Group-I | 91.00 ± 1.32 | 22.66 ± 0.62 | 33.66 ± 0.96 | 32.33 ± 0.33 | 1.65 ± 0.03 | 0.83 ± 0.06 |
| Group-II | 158.33 ± 1.23*** | 80.16 ± 0.65*** | 84.33 ± 1.05*** | 43.83 ± 0.70*** | 4.71 ± 0.04*** | 2.27 ± 0.08*** |
| Group-III | 138.66 ± 1.69*** | 86.00 ± 1.18*** | 90.17 ± 1.07*** | 37.83 ± 0.48*** | 3.40 ± 0.06*** | 1.62 ± 0.05*** |
| Group-IV | 177.16 ± 1.67*** | 96.16 ± 1.01*** | 102.0 ± 0.97*** | 46.66 ± 0.67*** | 6.68 ± 0.09*** | 4.25 ± 0.09*** |
| Group-V | 114.66 ± 1.63&&& | 43.33 ± 0.72&&& | 52.66 ± 0.96&&& | 37.33 ± 0.49&&& | 2.72 ± 0.10&&& | 1.52 ± 0.06&&& |
| Group-VI | 108.00 ± 1.16### | 36.16 ± 0.79### | 44.83 ± 1.12### | 40.83 ± 0.91## | 2.65 ± 0.09### | 1.09 ± 0.06### |
| Group-VII | 123.00 ± 2.21$$$ | 42.66 ± 0.88$$$ | 51.67 ± 0.72$$$ | 46.17 ± 0.65$$$ | 3.42 ± 0.10$$$ | 2.16 ± 0.07$$$ |
| Group-VIII | 109.50 ± 3.13&&& | 52.83 ± 0.87&&& | 62.50 ± 0.99&&& | 33.33 ± 0.84&&& | 2.22 ± 0.06&&& | 1.45 ± 0.12&&& |
| Group-IX | 108.16 ± 3.27### | 43.33 ± 0.66### | 52.83 ± 0.54### | 36.50 ± 0.43 | 1.53 ± 0.08### | 0.88 ± 0.06### |
| Group-X | 123.00 ± 1.98$$$ | 47.16 ± 0.79$$$ | 60.50 ± 0.85$$$ | 34.0 ± 0.36$$$ | 2.20 ± 0.06$$$ | 1.27 ± 0.06$$$ |

Values are mean ± SEM (N=6). ***$P$<0.001 as compare to control; &&&$P$<0.001 as compare to HFD; ##$P$<0.01, ###$P$<0.001 as compare to olanzapine; $$$$P$<0.001 as compare to olanzapine + HFD. One way ANOVA followed by Dunnett's multiple comparison tests.

a significant increase in blood glucose levels, AST, ALT, urea, uric acid, creatinine and lipid profile when compared to the control group. Curcumin and telmisartan significantly inhibited the HFD and olanzapine induced biochemical changes when compared to the HFD group and olanzapine, respectively. Like the HFD and olanzapine + curcumin combined group, the HFD and olanzapine + telmisartan combined group also significantly ($P<0.001$) inhibited the biochemical changes when compared to the HFD-fed group.

## DISCUSSION

The present study shows that treatment with curcumin and telmisartan prevents increase in body weight response to olanzapine and HFD-induced weight gain in rats. This suggests curcumin and telmisartan inhibited the olanzapine and HFD effects on body weight. According to our study results, it is possible to attenuate that antipsychotic-induced weight gain in SD rats was inhibited by ACE inhibitors and curcumin, a natural antioxidant.

The rats fed with normal diet did not exhibit excessive gain in body weight, but HFD-fed and olanzapine-administered animals showed significant increase in body weight. Shertzer et al. reported olanzapine-induced metabolic toxicity and the study report concludes that the olanzapine-induced weight gain was inhibited by the analgesic acetaminophen and by the antioxidant tetrahydroindenoindole.[24] A possible explanation for this finding may be found in previous studies where weight gain in humans and rodents treated with olanzapine was associated with single nucleotide polymorphisms (SNPs) in genes related to peripheral lipid homeostasis. In this way, the lipid deposing in adipose tissue may be proportional to the amount of fat consumption.[24,25] Like antioxidants, curcumin also acts on oxidative stress pathway and alters the disease state.

The animals treated with olanzapine and HFD showed significant increase in lipid levels suggesting that the olanzapine/second generation antipsychotic agents are the risk factors for hyperlipidemia and other cardiovascular diseases.[1] Olanzapine alone and olanzapine + HFD increased the triglyceride levels. It is a well known fact that olanzapine increases the body weight and serum triglyceride levels, as proved by various studies.[26] In telmisartan group, significant decrease in the relative organ weight of liver may be due to the reducing accumulation of visceral fat and decreased adipocyte size.[27]

Telmisartan is a known angiotensin II receptor antagonist that has partially agonistic properties on peroxisome proliferator-activated receptor (PPAR-γ).[28] The effects of telmisartan on insulin resistance and weight gain in genetic and nongenetic animal models was already reported. The earlier reports on effects of telmisartan on pioglitazone-induced increase in fat mass was modest in the SD rats and Zucker rats suggested that telmisartan did not interfere with the insulin-sensitizing properties of pioglitazone and attenuated the glitazone-induced increase in fat mass.[13]

In some cases, olanzapine caused metabolic syndrome by Pro12Ala polymorphism of PPAR-gamma2 and the polymorphism of PPAR might be important in olanzapine-induced weight gain.[29] High fructose feeding also altered lipid metabolism and decreased insulin sensitivity by suppression of hepatic PPAR-α.[30] The agent acting on the PPAR-γ receptor level needs to address the olanzapine and HFD-induced metabolic syndrome. Along with second generation antipsychotic drug treatment, addition of the natural antioxidant curcumin or partial PPAR-γ agonist telmisartan may helpful in controlling the olanzapine induced polymorphism on peroxisome proliferator-activated receptor to some extent. Thereby, the olanzapine/HFD metabolic syndrome/disease state will be altered.

The second generation antipsychotic drugs including olanzapine cause weight gain and obesity-related diseases result from excessive food intake and fat consumption. However, the rats were fed with HFD and treated with olanzapine mediated metabolic disorders, in part by altering the oxidative stress pathway. Curcumin is well known anti-oxidant, that may partially act as an uncoupling agent to increase the basal metabolism rate, thus reducing body weight gain and fat deposition.[24]

## CONCLUSION

The investigation was undertaken to study the effects of Curcumin and Telmisartan on olanzapine and HFD-fed induced insulin resistance. Thus, our results indicate curcumin and telmisartan affects the total lipid profile and glucose metabolism and favors the improvement of blood glucose and lipid profile. It also decreases body weight adipogenesis in liver. Further studies are needed to explore the underlying mechanisms.

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# Antithrombolytic and Antidiabetic Activity of Methanolic Extract of *Paederia foetida*

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## ABSTRACT

Present study was carried out to investigate the antidiabetic and antithrombolytic properties of methanolic extracts of the whole plant of *Paederia foetida*. Alloxan-induced male Sprague Dawley (SD) rats were given the whole plant extract of methanol by gastric gavage at doses of 250, 500, 1000 mg/kg body wt. and the results of reduction of blood glucose levels were compared with negative control (Tween 80 solution) & positive control (Glibenclamide). Methanolic extracts of the whole plant of *P. foetida* has moderate antidiabetic activity. At the 3rd hour, the dose of 250, 500 and 1000 mg/kg decreased the blood glucose levels by 31.6%, 28% and 30.3%, respectively, while the reference drug (glibenclamide, 2 mg/kg) decreased the blood glucose level by 70%. 100 µl extract of *P. foetida* exhibited the highest thrombolytic activity (among three samples) with clot lysis value of 23.82% whereas standard streptokinase (positive control) and water (negative control) demonstrated clot lysis value of 45.85% and 2.81%, respectively.

**Key words:** *paederia foetida* , antithrombolytic activity, antidiabetic activity, methanol extract

## INTRODUCTION

*Paederia foetida* belonging to family *Rubiaceae*[1] is one of 30 species in the genus *Paederia*. The origin of this plant is considered to be Eastern and southern Asian. It is usually found in different parts of India like Assam, Bihar and Orissa and also in Bangladesh. It possesses perennial twining vine from woody rootstock; stems to 7 m (23 ft) or more, climbing, or prostrate and rooting at the nodes. Leaves are opposite (rarely in whorls of 3), with conspicuous stipules. Petioles are commonly to 6 cm (2.4 in) long; blades entire, oval to linear-lanceolate, 2-11 cm (1-4.3 in) long, hairy or glabrous, often lobed at base. The leaves and stems have disagreeable odor, especially when crushed. Flowers are small, grayish pink or lilac, in broad or long, "leafy" curving clusters, terminal or at leaf axils. Corolla are densely hairy, tubular with 5 (usually) spreading lobes. Fruits are shiny brown, nearly globose capsule, to 0.7 cm (0.3 in) wide, with 2 black roundish seeds, often dotted with white raphides. Many traditional plants are used for thrombolytic and antidiabetic activity throughout the world.[2] Plant drugs[3] and herbal formulations[4,5,6] are frequently considered to be less toxic and freer from side effects than the synthetic one.

Streptokinase, an antigenic thrombolytic agent, is used for the treatment of acute myocardial infarction. In most infarct patients, it reduces mortality as effectively as the nonantigenic altreplase while having the advantages of being much less expensive. Tissue-type Plasminogen activator (tPA) is generally preferred as being effective and safer than either urokinase or streptokinase type activators. All available thrombolytic agents still have significant shortcomings. In some cases, large doses are required to be maximally effective, have limited fibrin specificity and a significant associated bleeding tendency. Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs.[7,8,9,10,11] The present study was done to measure the thrombolytic activity of methanol extract of the whole plant of *P. foetida* by using streptokinase as a reference standard.

Diabetes is a serious metabolic disorder with micro and macro vascular complications which causes significant morbidity and mortality. In the developing world, the diabetes epidemic is accelerating with an increased

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proportion of affected people in the younger population. Recently, some reports described that type-2 diabetes was being diagnosed even in children and adolescents.[12] The latest WHO Global Burden of Disease estimates the worldwide burden of diabetes in adults to be around 173 million in the year 2002[13] and around two-thirds of these live in developing countries. So, now-a-days, it has become a growing public health concern worldwide causing severe and costly complications including blindness, cardiac and kidney diseases.[14] Current therapies do little in preventing complications although they provide good glycemic control. Besides this, these drugs are also associated with side effects. Thus, it is necessary to continue research for new and, if possible, more efficacious drugs. Based on the WHO recommendations, hypoglycemic agents of plant origin used in traditional medicine are important.[15] No study has been carried out on the antidiabetic activity of *Paederia foetida* leaves. Therefore, the current study focuses on the antidiabetic potentiality of methanol extracts of the whole plant of *P. foetida*.

## MATERIALS AND METHOD

### Plant materials

The whole plant of *Paederia foetida* was collected from Ishurdi, Pabna and was taxonomically identified with the help of the National Herbarium of Bangladesh. Accession Number of the plant is 34418. The whole plants were cut into small pieces and then sun dried for seven days. The dried plants were then ground into coarse powder with the help of an attrition type of a grinder.

### Extraction and isolation

Extraction of dried and powdered plant of *P. foetida* was done by the extraction process by using methanol as a solvent.[16] The air-dried and pulverized plant material was extracted with methanol. After that the fractions were evaporated by roto-dryer to dryness at low temperature (40-50 °C). Crude methanol extract was subjected to evaluate thrombolytic and antidiabetic activity.

### Experimental animal

Male Sprague Dawley rats, aged 5-6 weeks, were collected from the animal research branch of the International Center for Diarrheal Disease and Research, Bangladesh (icddr,b). Animals were maintained under standard environmental conditions (temperature: [24.0 ± 1.0 °C], relative humidity: 55-65% and 12 h light/dark cycle) and had free access to feed and water *ad libitum*. The animals were acclimatized to laboratory conditions for one week prior to the experiments. Principles of laboratory animal care guidelines (NIH publication number 85-23, revised 1985) were followed.

### Thrombolytic activity

5 ml of blood sample was collected from a healthy volunteer and was distributed into five separate pre-weighed ($W_1$) microcentrifuge tubes. The tubes were centrifuged at 2500 rpm for five minutes and then incubated for 45 minutes at 37 °C. After clotting of blood, serum was decanted and removed. Then weight of clotted blood (ΔW) was taken by subtracting the pre-weight ($W_1$) from the weight of the clot containing tube (W2) as: $\Delta W = W_2 - W_1$. Then 100 µl extract of *P. foetida* was added to the clot containing tube. Similarly 100 µl of streptokinase was added to clot of standard tube and 100 µl of water was added to clot of blank tube, which were used as positive and negative control, respectively. Then all test tubes were incubated at 37 °C for 90 minutes and weighed again for getting the weight variation among the pre-weight and final weight ($W_3$) that was achieved for clot lyses (thrombolysis). Average value of weight loss was calculated in percentage (%) of clot lysis which was calculated with the following formula :

$$\% \text{ of clot lysis} = \left(\frac{\text{wt. of released clot}}{\text{clot wt.}}\right) \times 100\% = \left(\frac{W_2 - W_3}{W_2 - W_1}\right) \times 100\%$$

### Anti-diabetic study

Alloxan monohydrate (Sigma-Aldrich, USA) solution of 10 mg/ml was prepared in 0.1M ice-cold citrate buffer (pH 4.5) and then administered to the rats within 5 mins at a dose of 50 mg/kg bodyweight intraperitonially. The fasting blood sugar levels of each of the rats were checked everyday with an autoanlyzer (Glucometer, Bioland G-423 S) glucose kit. After 8 days, animals with fasting blood sugar levels of 250 mg/dl and above were considered to be diabetic and were used for the study. All the selected rats were divided into five groups of five rats each. Group I served as the negative control and received tween-80 solution (solvent used to dissolve the extract) at the rate of 10 ml/kg body weight. Groups II, III & IV received the *P. foetida* extract at the dose of 250, 500 and 1000 mg/kg respectively while group V served as the positive control and received the standard reference drug glibenclamide (5 mg tablet of Daonil from Sanofi-Aventis) 5 mg/kg all by gastric gavage. The blood glucose levels of the rats were measured at 0, 1, 2 and 3 h after administration of drug and extracts. Blood samples were collected by tail snip and the blood glucose measured with an autoanalyzer (Glucometer, Bioland G-423 S) glucose kit.[17] At the end of the experiment, percentage reduction of the glucose levels of the rats at the 3rd hour was calculated using the formula below:

$$\text{Percentage Reduction} = \left[\frac{(\text{BGL at 0 hr} - \text{BGL at 3}^{\text{rd}}\text{ hr})}{\text{BGL at 3}^{\text{rd}}\text{ hr}}\right] \times 100\%$$

BGL = Blood Glucose Level

**Table 2: Effect of *Paederia foetida* on the fasting blood glucose levels of alloxan-induced diabetic rats**

| Group | Treatment | Fasting Blood Glucose Level (mg/ml) | | | | % reduction at the 3rd hour |
|---|---|---|---|---|---|---|
| | | 0 hr | 1 hr | 2 hr | 3 rd | |
| I | Alloxan ± Tween80 (1%) | 387 ± 5.1 | 398.5 ± 4.3 | 397.8 ± 3.7 | 397.6 ± 3.3 | - |
| II | Alloxan ± PFE (250 mg/kg) | 365.8 ± 5.3 | 222.3 ± 3.9 | 245.7 ± 5.1 | 250.2 ± 4.2 | 31.6 |
| III | Alloxan ± PFE (500 mg/kg) | 389.7 ± 4.8 | 337.6 ± 3.3 | 314.5 ± 3.7 | 280.4 ± 6.2 | 28 |
| IV | Alloxan ± PFE (1000 mg/kg) | 355.2 ± 4.7 | 313.6 ± 3.4 | 275.1 ± 5.0 | 247.4 ± 4.5 | 30.3 |
| V | Alloxan ± Glibenclamide (5 mg/kg) | 344.3 ± 1.0 | 172.7 ± 1.1 | 115.9 ± 1.9 | 103.6 ± 0.7 | 70 |

$p < 0.001$ was considered statically significant, PFE = *P. foetida* extract

**Table 1: Thrombolytic activity of methanolic extract of *Paederia foetida***

| No. of sample | Weight of empty test tubes, $W_1$ | Weight of blood clot test tubes, $W_2$ | Weight of release clot test tubes, $W_3$ | % of clot lysis |
|---|---|---|---|---|
| [a]PFS-1 | 6.0279 | 6.1539 | 6.1286 | 20.08 |
| PFS-2 | 6.0450 | 6.3203 | 6.2547 | 23.82 |
| PFS-3 | 6.0599 | 6.2470 | 6.2123 | 18.55 |
| Water | 6.0560 | 6.1425 | 6.1027 | 45.85 |
| Streptokinase | 6.0321 | 6.2416 | 6.2357 | 2.81 |

[a]PFS = *Paederia foetida* sample

## RESULTS

### Thrombolytic activity

The percentage of weight loss of clot after application of the extract solution was taken as the functional indication of thrombolytic activity. The study was implemented on one healthy volunteer with five blood samples (Table 1).

### Antidiabetic Activity of Diabetic Induced Rats

The effect of the methanolic extracts of the whole plant of *P. foetida* on the fasting blood glucose levels of alloxan-induced diabetic rats is presented in Table 2.

## DISCUSSION

The comparison of positive and negative control as shown in Table 1 clearly stated that clot dissolution doesn't occur when water was added to the clot. On the basis of our study, compared to the value of standard (45.85%), the average % of clot lyses was 20.82 ± 2.71 which is approximately half of the standard. So, it can be said that the methanolic extract of the whole plant has moderate thrombolytic activity.

The result showed that there was no significant change in the blood glucose levels of rats in group I that received tween-80 solutions (negative control). The methanolic extract of *P. foetida* in all the doses used, including the reference drug, caused a time dependent and significant ($p < 0.001$) reduction of the blood glucose levels of the alloxan-induced diabetic rats when compared to the negative control group. The highest activity of *P. foetida* extract in this experiment was observed at the dose of 250 mg/kg while the reference drug glibenclamide (5 mg/kg) had a superior activity.

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# Comparative *in vitro* Antimicrobial and Phytochemical Evaluation of Methanolic Extract of Root, Stem and Leaf of *Jatropha curcas* Linn

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## ABSTRACT

**Background:** Earlier researchers have reported antibacterial activity of different specific parts, but none of the reports show the comparative microbial and phytochemical studies of root, stem and leaf extract. **Objective:** To compare and investigate antimicrobial, qualitative phytochemical studies, phenol, flavonoid and TLC analysis of root, stem bark, leaf extracts of *Jatropha curcas* Linn family Euphorbiaceae. **Methods:** The dried plant powder was subjected to Soxhlet extraction with methanol. These solvent extracts were subjected to a preliminary phytochemical screening to detect the different chemical principles present viz., carbohydrates, proteins, amino acids, steroids, glycosides, alkaloids, flavonoids, tannins and phenolic compounds, fixed oils. Antimicrobial activity was evaluated by disc diffusion method and minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), minimum fungicidal concentration (MFC) was calculated by micro dilution method. Thin layer chromatography was also performed using solvent system chloroform, benzene, hexane, and ethyl acetate for the analysis of a number of constituents in the plant extract. The content of the total phenolics in the extract was determined spectrometrically according to the Folin-Ciocalteu procedure and calculated as catechol equivalent. The content of total flavonoids in the extract was determined and calculated as quercetin equivalent. **Result:** These extracts showed antibacterial, antifungal activities against gram-positive and gram-negative bacteria with varying magnitudes. The phytochemical analysis showed the presence of alkaloid, saponin, tanins, terpenoids, steroids, glycosides, phenols and flavonoids. Maximum phenolic content (38.8) was found in leaf extract and flavonoid content (18.14) in latex of plant. **Discussion:** It is concluded that the antimicrobial activity showed by the plant was due to the presence of these phytochemicals. Further studies are highly needed for drug development.

**Key words:** phytochemical, microdilution, TLC, folin-ciocalteu, quercetin, MBC, MFC.

## INTRODUCTION

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well being. Their role is two fold in the development of new drugs. They may become the base for the development of a medicine, a natural blue print for the development of new drugs or a phytomedicine to be used for the treatment of diseases.[1] Traditional medicine using plant extracts continues to provide health coverage for over 80% of the world's population, especially in the developing world.[2] *Jatropha* species belongs to the family Euphorbiaceae and is used in traditional folklore medicine to cure various ailments in Africa, Asia and Latin America.[3] *Jatropha curcas* Linn is commonly called physic nut, purging nut or pig nut. Previous studies have reported that the plant exhibits bioactive activities as wound healing,[4] antidiarrhoeal,[5] antidiabetic,[6] antitumor effects,[7] immunomodulatory activity[8] and used

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in treatment of rheumatism.[9,10] Fagbenro-Beyioku (1998) investigated and reported the anti-parasitic activity[11] of the sap and crushed leaves of *J. curcas*. The water extract of the branches also strongly inhibited HIV-induced cytopathic effects with low cytotoxicity.[12] Previous works have shown that many *Jatropha* species possess antimicrobial activity[13] and antifungal activity of *J. curcas* seed cake.[14] Many useful products from seeds, especially the oil which is extracted from the seed is of multipurpose use.[15,16] In addition, PEs with molluscicidal/antimicrobial activities could be utilized for agricultural and pharmaceutical[17] use with antihelminthic activity.[18] After extraction of oil, *Jatropha* presscake strongly supports its use as a potential coagulant agent ,[19] which can also be utilized as a substrate for biogas production.[20] Defatted *Jatropha curcas* L. (*J. curcas*) seed kernels contained a high percentage of crude protein (61.8%) and relatively little acid detergent fiber (4.8%) and neutral detergent fiber (9.7%), recommending its use as a source of anticancer therapeutic agent toward breast cancer cells.[21] Several studies have confirmed the antimicrobial efficacy of different *Jatropha* species. Whatever limited information is available on the medicinal properties of *Jatropha curcas* is mostly on the leaf extracts of the plant.

Therefore this study was planned to evaluate comparative antibacterial and antifungal activity of crude extracts of the stem bark, root and leaf of *J. curcas* and also investigate preliminary phytochemical analysis including phenolic and flavonoid content.

## MATERIAL AND METHODS

### Plant Material and Extraction

The plant *Jatropha curcas* Linn was collected from Botanical Garden of the Institute of Agriculture Science, Banaras Hindu University, Varanasi (India). The identification of the plants was done by Dr. K.N. Dwivedi, Department of Dravyaguna, Faculty of Ayurveda, Institute of Medical Sciences, Banaras Hindu University, Varanasi (India). Reference number "DG/KND/11-12/603" was given to the plant sample.

### Preliminary Phytochemical Screening

Chemical tests were carried out on the alcoholic extract and on the powdered specimen using standard procedures to identify the constituents.[22,23] The plant extract was assayed for the presence of alkaloids, glycosides, flavonoids, tannins, phenolic compounds, saponins, terpenoids and steroids.

#### *Testing for alkaloids*

Each extract (0.5 g) was stirred with 5 mL of 1% HCL on a steam bath. The solution obtained was filtered and one mL of the filtrate was treated with a few drops of Mayer's reagent. The turbidity of the extract filtrate on addition of Mayer's reagent was taken as evidence of the presence of alkaloids in the extract.

#### *Testing for tannins and phenolics*

Each extract (0.5 g) was separately stirred with 10 ml of distilled water and then filtered. Aew drops of 5% $Fecl_3$ reagent were added to the filtrate. Blue-black or blue green colouration or precipitation was taken as an indication of the presence of phenolics and tannins.

#### *Test for saponin*

About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered, 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, and observed for the formation of emulsion.

#### *Test for steriods*

Two ml of acetic anhydride was added to 0.5 ethanolic extract of each sample with 2 ml $H_2S0_4$. The colour changed from violet to blue or green in some samples indicating the presence of steroids.

#### *Test for terpenoids (Salkowski test)*

Five ml of each extract was mixed in 2 ml of chloroform, and concentrated $H_2S0_4$ (3 ml) was carefully added to form a layer. A reddish brown colouration of the inter face formed to show positive results for the presence of terpenoids.

#### *Test for cardiac glycosides (Keller-Killani test)*

Five ml of each extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring of interface indicated the deoxysugar characteristic of cardenolides. A violet ring could appear below the brown ring, while in the acetic acid layer, a greenish ring formed just gradually throughout the thin layer.

### Screening of Antibacterial Activity

#### *Test Microorganism*

A total of 4 bacterial strains viz. *S. flexineri* ATCC 12022, *E. coli* ATCC 25922(Gram-negative), *S. aureus* ATCC 25323, *E. faecalis* (gram- positive), and four fungal strains namely *Candida albicans* ATCC 90028, *Candida krusei* ATCC 6258, *Candida tropicalis* ATCC 750, *Candida parapsilosis* ATCC 22019 were used in the investigation. All cultures were obtained from the American Type Culture Collection (ATCC), MTCC, clinical strains preserved at Department of Microbiology, Institute of Medical Sciences, BHU, Varanasi, India. Fresh bacterial broth cultures were prepared before the screening procedure.

### Preparation of sample extract for microbiological assay

About 1g of each extract was dissolved in 10 ml (100 mg $ml^{-1}$) of peptone water to obtain a stock solution and the working solution was prepared. The extract was diluted as 1:10 equivalent to 100 mg $ml^{-1}$ and 1:5 dilution equivalent to 50 mg $ml^{-1}$, from which 5 µl was dispensed on a sterile disc of Whatman's filter paper no. 1 of 6 mm diameter for susceptibility testing.

### Antimicrobial Susceptibility Test

The disc diffusion method was used to screen the antibacterial activity[24,25,26] and antifungal activity.[27,28] Muller Hinton agar (MHA) plates were prepared by pouring 15 ml of molten media into sterile petriplates. The fresh grown bacteria was suspended in sterile saline to achieve concentration of $10^7$ cfu/ml. This suspension was spread on the surface of MHA agar plates. The plates were allowed to dry for 5 min. The different concentrations of extract (100, 200 mg/ml) were put on 6 mm sterile disc of Whatman filter paper no. 1. The disc was then placed on the surface of the medium and the compound was allowed to diffuse for 5 min and the plates were kept for incubation at 37 °C for 24 hr for bacteria and 48 hr at 25 °C for fungal agents. At the end of incubation, inhibition zones were examined around the discs, which if present, were measured with transparent ruler in millimeters. This study was performed in triplicate.

### Determination of MIC, MBC and MFC

MIC was determined by micro-dilution method[29,30] using serially diluted (2 fold) plant extracts according to the National Committee for Clinical Laboratory Standards (NCCLS) (National Committee for Clinical Laboratory Standards, 2000).[31] MIC of the extracts was determined by dilution of methanolic extract with various concentrations. Equal volume of each extract and nutrient broth were mixed in wells of microtiter plate. Specifically 0.1 ml of standardized inoculums (1-2 × $10^7$cfu/ml) was added in each tube. The plates were incubated aerobically at 37 °C for 18-24 h for bacteria and 48 h at 25 °C for fungal growth. Two control wells were maintained for each test batch. These included antibiotic control (containing extract and growth media without inoculum) and organism control (tube containing the growth medium, saline and the inoculum). The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control were regarded as MIC. However, the MBC and MFC were determined by sub-culturing the test dilution on to a fresh drug free solid medium and incubated further. The highest dilution that yielded no bacterial or fungal colony was taken as MBC and MFC.

### Media used

Muller-Hinton agar, Luria broth (Hi-media, Mumbai, India), and Sabouraud dextrose agar pH 7.3 ± 0.2 (Hi-media), RPMI 1640 were used for antibacterial and antifungal activity respectively.

### Determination of Total Phenols (Tp) by Spectrophotometric Method

Total Phenolic concentration in different fractions of alcoholic extract was measured by Folin Ciocalteau assay. Briefly, 5 ml of distilled water, 0.5-1.0 ml of sample, and 1.0 ml of Folin Caiocalteau reagent was added to a 25 ml flask. The content was mixed and allowed to stand for 5-8 min at room temperature. Next 10 ml of 7% sodium carbonate solution was added followed by distilled water. Solution was mixed and allowed to stand at room temperature for 15 min, and then absorbance was recorded at 750 nm. TP content was standardized against gallic acid and expressed as milligram per liter of gallic acid equivalents (GAE). The linearity range for this assay was determined as 0.5-5.0 mg/l GAE ($R^2$=0.999), giving an absorbance range of 0.050-0.555 absorbance units.[32,33]

### Determination of Total Flavonoid Content

Total flavonoid content was measured by using aluminium chloride (2%) in which it was mixed with a solution of the test samples. Absorbance reading at 415 nm (Elico SL 177) was taken after 10 min against a blank sample consisting of 5 ml of sample solution and 5 ml of methanol without aluminium chloride. The total flavonoid content was determined using a standard curve of quercetin at 0-50 µg/ml. The average of three readings was used and then expressed in µg quercetin equivalent flavones per mg extract.[33]

#### *Thin layer chromatography (TLC)*

Thin layer chromatography (TLC) was used to separate the different part of *Jatropha curcas* extract into different spots on the chromatplate. The chromatograms developed on the microscope slide, were dried and observed visually for the different parts of plant extract components. The developing solvents used in different extracts were hexane, chloroform, and benzene with ratio 9:1.

The retention factor was calculated using the following equation:

$$Rf = \frac{\text{Distance move by the substance (cm)}}{\text{Distance move by the solvent (cm)}}$$

## RESULT

Ancient Indian system of medicine (Ayurveda) is mainly based on herbal treatment. The root, stem, bark, leaf of *J. curcas* Linn are extensively used in the management of

various infections and allergic diseases. The preliminary phytochemical screening of alcoholic extract of various parts of *J. curcas* Linn is presented in Table 5, showing the presence of alkaloids, phenolic groups, flavonids, saponins, steroids, tannins, cardiac glycosides and terpenoids. In addition to the phytochemical screening, antimicrtobial efficacy was determined on the basis of number of secondary metabolites. Activities of extracts

**Table 1: Determination of MIC, MBC (mg/ml) of *Jatropha Curcas* Linn**

| Strains | *E. coli* ATCC 25922 | | *S. aureus* ATCC 25323 | | *E. faecalis* | | *S. flexineri* ATCC 12022 | |
|---|---|---|---|---|---|---|---|---|
| Extract | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| ROOT | 20 | 25 | 6.25 | 6.25 | 10 | 12.5 | 12.5 | 25 |
| LEAF | 12.5 | 12.5 | 12.5 | 25 | 6.25 | 6.25 | 6.25 | 12.5 |
| STEAM | 10 | 12.5 | 6.25 | 12.5 | 10 | 12.5 | 10 | 12.5 |

**Table 2: Determination of MIC, MFC (mg/ml) of *Jatropha curcas* Linn**

| Strains | *C. albicans* ATCC 90028 | | *C. tropicalis* ATCC 750 | | *C. krusei* ATCC 6258 | | *C. parapsilosis* ATCC 22019 | |
|---|---|---|---|---|---|---|---|---|
| Extract | MIC | MFC | MIC | MFC | MIC | MFC | MIC | MFC |
| ROOT | 12.5 | 25 | 12.5 | 25 | 25 | 30 | 20 | 25 |
| LEAF | 10 | 12.5 | 12.5 | 30 | 12.5 | 20 | 12.5 | 12.5 |
| STEAM | 12.5 | 25 | 25 | 30 | 12.5 | 25 | 20 | 25 |

**Table 3: TLC Result of root, stem and leaf methanolic extract of *Jatropha curcas* Linn**

| Extracts | Solvent system | Number of components | Distance of spot (cm) | Solvent front (cm) | Rf value |
|---|---|---|---|---|---|
| Root | Benzene:chloroform:few drop acetic acid (9:1) | 4 | 8.9, 7.3, 6.9, 5.4, | 10.9 | 0.81, 0.66, 0.63, 0.49 |
| Stem | Hexane:chloroform:few drop acetic acid(9:1) | 5 | 8.8, 8.1, 7.3, 4.5, 2.9 | 10.2 | 0.86, 0.79,0.70, 0.44, 0.26 |
| Leaf | Hexane:chloroform:few drop acetic acid (9:1) | 4 | 11.5, 9.3, 7.1, 3.4 | 12.5 | 0.92, 0.74, 0.56, 0.27 |

**Table 4: Antimicrobial activity of the different extract of *Jatropha curcas* Linn plant**

| Microorganism | Zone of inhibition (in mm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | *J.curcas* extract concentration ( mg/ml) | | | | | | Standard drugs |
| | Root | | Leaf | | Steam | | |
| | 100 | 200 | 100 | 200 | 100 | 200 | |
| ***S. flexineri* ATCC 12022** | 7 ± 0.19 | 9 ± 0.29 | 12 ± 0.53 | 14 ± 0.73 | 10 ± 0.32 | 12 ± 0.52 | 25 (Ciprofloxacin) |
| ***E. coli* ATCC 25922** | 6 ± 0.11 | 9 ± 0.21 | 8 ± 0.11 | 10 ± 0.32 | 9 ± 0.59 | 12 ± 0.79 | 26 (Norfloxacin) |
| ***S. aureus* ATCC 25323** | 9 ± 0.12 | 11 ± 0.24 | 11 ± 0.17 | 13 ± 0.37 | 8 ± 0.35 | 10 ± 0.55 | 24 (Ampicilin) |
| ***E. faecalis*** | 9 ± 0.33 | 11 ± 0.55 | 10 ± 0.11 | 12 ± 0.27 | 8 ± 0.25 | 10 ± 0.81 | 28 (Ciprofloxacin) |
| ***C. albicans* ATCC 90028** | 8 ± 0.36 | 10 ± 0.56 | 8 ± 0.42 | 10 ± 0.62 | 7 ± 0.50 | 9 ± 0.43 | 16 (Fluconazole) |
| ***C. krusei* ATCC 6258** | 6 ± 0.54 | 8 ± 0.74 | 10 ± 0.25 | 12 ± 0.45 | 8 ± 0.15 | 10 ± 0.27 | 18 (Fluconazole) |
| ***C. tropicalis* ATCC 750** | 10 ± 0.27 | 12 ± 0.47 | 9 ± 0.26 | 11 ± 0.36 | 7 ± 0.73 | 9 ± 0.41 | 16 (Amphotericin B) |
| ***C. parapsilosis* ATCC 22019** | 7 ± 0.18 | 9 ± 0.38 | 10 ± 0.24 | 12 ± 0.44 | 7 ± 0.36 | 9 ± 0.39 | 15 (Amphotericin B) |

**Table 5: Phytochemical analysis of various parts of *Jatropha curcas* Linn using methonolic extract**

| S. No. | Constituents | Tests | Leaf extract | Root extract | Stem extract |
|---|---|---|---|---|---|
| 1 | Alkaloids | Mayer's reagent | + | + | + |
| 2 | Saponin | Foam test | + | + | + |
| 3 | Tanin | Extract + 5% Fecl3 | + | + | + |
| 4 | Terpenoid | Salkowski test | + | – | + |
| 5 | Steroid | Liebermann-Burchard reaction | + | + | + |
| 6 | Glycosides | Keller-kilani test | + | + | + |
| 7 | Phenolic Compound | Extract + 5% Fecl3 | + | + | + |
| 8 | Flavonoid | Residue + Lead acetate soln. | + | + | + |

against test organism were expressed in the form of diameter of zone of inhibition around extract (Table 4). The inhibition zone increases with increase in concentration. The MIC of root, leaf and stem extract for different organism ranged between 6.25 mg/ml and 25 mg/ml. The MBC and MFC of the various extracts for different microbes ranged between 6.25 mg/ml and 30 mg/ml (Tables 1 and 2). The results TLC analysis using benzene: hexane: chloroform solvent mixture as shown in Table 3 revealed four spots for root, five spots for stem and four spots for leaf extracts.

Estimation of total phenolic and total flavonoid content showed that the leaf extract was having the maximum phenolic content (38.8 ± 2.14) in µg Gallic acid equivalents (GAE) followed by the root extract (26.15 ± 3.84) with a very less flavonoid content in root (1.88 ± 1.00) and leaf (1.72 ± 2.08) extracts in µg of quercetin equivalents (QE).

## DISCUSSION

These classes (alkaloids, saponins, tannins, anthraquinones and flavonoids) of compounds are known to have activity against several pathogens and therefore aid the antimicrobial activities of *J. curcas* and suggest their traditional use for the treatment of various illness.[34,35] In all the three extracts, tannins were present resulting in the inhibition of cell protein synthesis as it forms irreversible complexes with prolinerich protein.[36] Tannin containing herbs have been reported in treating intestinal disorders such as diarrhea and dysentery,[37] treatment of inflamed or ulcerated tissues.[38] *In vitro* antibacterial test results presented in Figure 1 show antibacterial activity against gram-positive and negative bacteria with strong antifungal activity (Figure 2). The methanolic extracts exhibited considerable level of inhibition against the entire test organism compared to the standard drug. This is suggestive of the presence of some compounds or groups in the extract with similar mechanism of action to that of the standard drug used in bacterial and fungal activity. It has been observed that tannins have anticancer activity and can be used in cancer prevention, thus suggesting that *J. curcas* has potential as a source of important bioactive molecules for the treatment and prevention of cancer. The presence of tannins in *J. curcas* supports the traditional medicinal use of this plant in the treatment of different ailments. These observations therefore support the use of *J. curcas* in herbal cure remedies.

Various workers have already shown that gram-positive bacteria are more susceptible towards plants extracts as compared to gram negative. These differences may be attributed to the fact that the cell wall in gram-positive bacteria is of a single layer, whereas the gram negative cell wall has a multilayered structure. Alternatively, the passage of the active compound through the gram negative cell wall may be inhibited. Although *J. curcas* leaf, root and stem extracts show less MIC against gram positive as compared to gram negative bacteria. It is thought that the observed differences may result from the doses used in this study. In addition, microorganisms show variable sensitivity to chemical substances related to different resistance levels between strains. Mujumdar et al. (2001) also reported that the crude methanol extract from the root of *J. curcas*

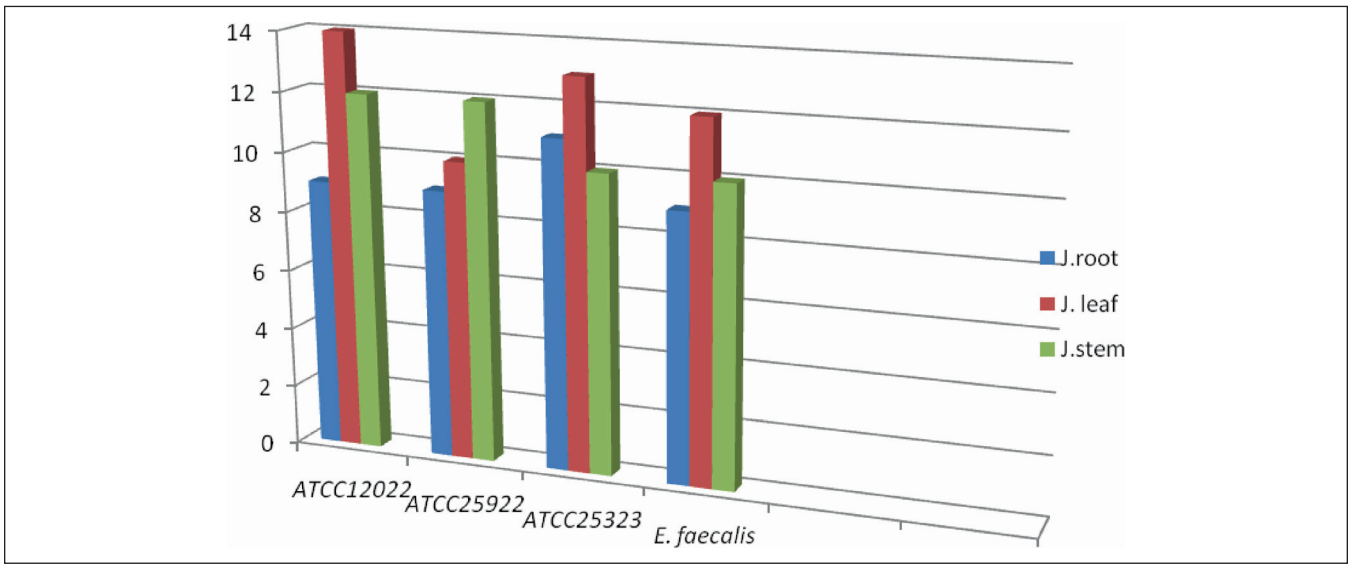


**Figure 1:** Antibacterial effect (in mm) of root, stem and leaf extract
*Graph showing antibacterial activity with various strains, among root, stem and leaf. Leaf extract of J. curcas is comparatively more active in all the microorganisms as leaf extract has a higher zone of inhibition than the root and stem bark.*

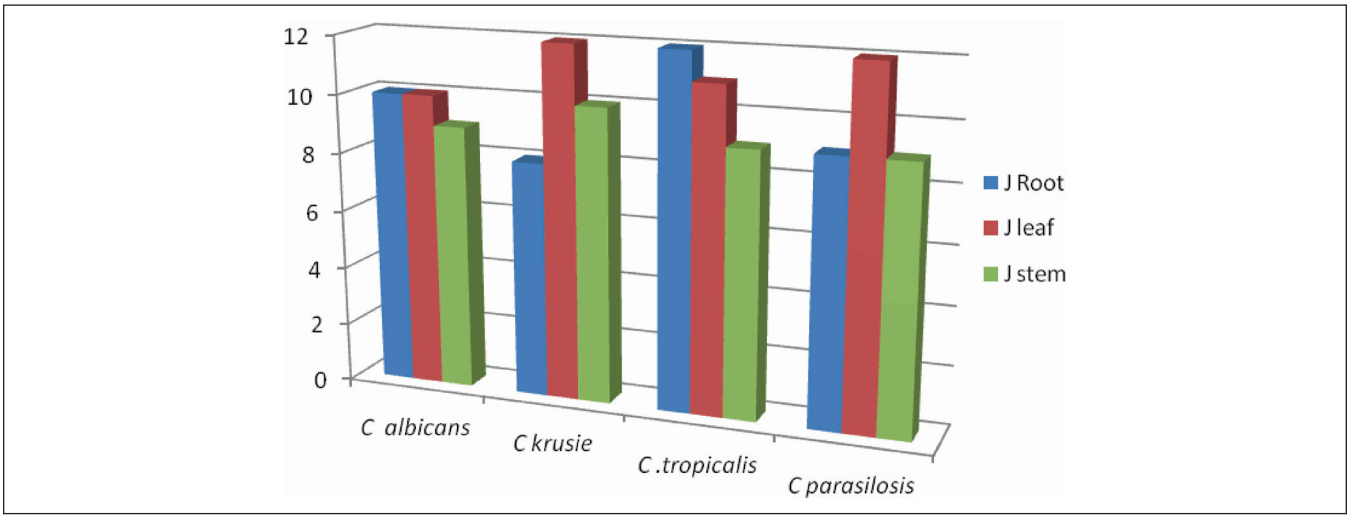


**Figure 2:** Antifungal effect (in mm) of root, stem and leaf extract
*Graph showing antifungal activity with various strains, among root, stem and leaf. Leaf extract of J. curcas is comparatively more active in all candida species as leaf extract has a higher zone of inhibition than root and stem bark.*

exhibited anti-diarrhea activity[39] in mice through the inhibition of prostaglandin biosynthesis and the reduction of osmotic pressure. Recently, Aiyelaagbe et al. (2007) reported that the presence of some secondary metabolites in the root extract of *J. curcas* inhibited some microorganisms isolated from sexually transmitted infections.[40] This may be attributed to the presence of soluble phenolic and polyphenolic compounds.[41] However, it may be suggested that plant extracts exhibiting diameters of zones of inhibition larger than 10 mm are considered active.[35] Thus it is believed that the extract is a better antimicrobial agent for various pathogenic fungus and bacteria. Among these, leaf extract was showing better antibacterial and antifungal properties compared to stem and root extract of *J. curcas*. The pharmacological activities of the drug may be contributed to the presence of secondary metabolites.

Hence, the presence of some metabolites in *J. curcas* suggests its activities against microbes. It is concluded that leaf, stem and root of *J. curcas* could be a potential source of active antimicrobial agents, and a detailed assessment of its *in vivo* potencies and toxicological profile is ongoing.

## CONCLUSION

The inhibitory effect of the extract of *J. curcas* against pathogenic bacterial strains can introduce the plant as a potential candidate for drug development for the treatment of ailments caused by human pathogens. The ability of the extracts to inhibit the growth of several bacterial and fungal species is an indication of the broad spectrum anti-microbial potential of various parts of *J. curcas*, which makes the complete plant a candidate for bioprospecting for antibiotic and antifungal drugs.

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## CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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# Evaluation of Antinociceptive, Antidiarrheal and Antimicrobial Activities of Leaf Extracts of *Clerodendrum indicum*

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## ABSTRACT

**Introduction:** The methanolic extracts and its different partitioning fractions of leaves of *Clerodendrum indicum* were evaluated for their anti-nociceptive, anti-diarrheal and *in vitro* antimicrobial activities. **Methods:** The anti-nociceptive activity was evaluated using the acetic acid-induced writhing test in mice; the anti-diarrheal activity was investigated by the effect of extracts on castor oil-induced diarrhea while the *in vitro* antimicrobial activities were examined by the disc diffusion method. *Results:* In the acetic acid-induced writhing test, the methanolic extract at a dose of 200 and 400 mg/kg showed a significant ($p$<0.001) and dose-dependent reduction in the number of writhes with 62.57% and 70.76% of inhibition, respectively, while the $CCL_4$ fraction at the same dose showed potent anti-nociceptive activity ($p$<0.001) with 73.09% of inhibition of writhing which was even higher than that of standard diclofenac sodium (55.56% inhibition). The methanolic extract, $CCL_4$ and chloroform fraction showed moderate activity against the tested microorganisms in terms of both zones of inhibition (ranged from 9-13 mm, 10-13 mm and 10-13 mm, respectively, at a concentration of 400 µg/disc) and spectrum of activity. In castor oil-induced diarrhea testing, the methanolic extract and chloroform fraction at a dose of 400 mg/kg produced 21.74% and 26.96% inhibition of defecation, respectively, which were found to be comparable to that of standard drug loperamide (37.39% inhibition at 50 mg/kg ) with regard to the severity of diarrhea. **Conclusion:** The results of the investigation demonstrated that the methanolic extract and its different fractions of leaves of *Clerodendrum indicum* possess significant anti-nociceptive, antimicrobial and antidiarrheal activities.

**Key words:** *Clerodendrum indicum*; leaves; anti-nociceptive; antimicrobial; anti-diarrheal; writhing

## INTRODUCTION

Since ancient times, medicinal plants have been used for the treatment and management of various health problems. About 80% of the world's population relies on the use of traditional medicine, which is predominantly based on herbal products.[1] To ensure the rational use of herbal medicine, it is imperative to validate the folkloric claim of medicinal plants used in traditional medicine so that the beneficial ones can be deployed as phytomedicines and the bioactive constituents from such beneficial plants could be isolated and used as "leads" in drug discovery process.[2]

*Clerodendrum indicum* (family: Verbenaceae; vernacular names: Bamunhatti, Nuli gach) is an annual shrub which is found in areas with moderate temperature. The species occurs variably in India, Nepal, Myanmar, Malaya, Indo-China, Indonesia, Java and Bangladesh. Leaves (aerial parts) and roots of *Clerodendrum indicum* are used for various medicinal purposes. In traditional system of remedies, the plant is mainly used in the treatment of asthma, bronchitis, cold fever, intestinal worms, arthritis, epilepsy, febric convulsion, gastric tumor, hematuria, hysteria, impotence, lipoma, nasal polips, painful micturation and rheumatism.[3-5] Paste made out of its leaves is effective in application on wounds for early healing,

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and healing of the infected lymph node (lymphadenopathy). Leaf powder is used in digestive disorders and other GI-related ailments. It purifies blood, improves blood circulation and suppresses all kind of swelling of the body. It acts on the respiratory system thus expelling out the excessive mucus in the tract relieving cough, cold and asthma symptoms.

Although the leaves of the plant have been traditionally used in the treatment of various painful and anti-inflammatory conditions, gastrointestinal disorders and infectious disease, there is no extensive anti-nociceptive, antimicrobial and anti-diarrheal study of this valuable medicinal plant. Only Raihan et al.[6] reported the analgesic activity of crude ethanolic extract of leaves and Rahman et al.[7] reported the *in vitro* antibacterial activity of root and stem of the plant previously. To prove the ethno-medical claims, the present study was designed to evaluate the anti-nociceptive and anti-diarrheal activities of the methanolic extracts and its different partitioning fractions of leaves of *Clerodendrum indicum* in mice model. The *in vitro* antimicrobial activities of the methanolic extracts and its different partitioning fractions of leaves were also investigated.

## MATERIALS AND METHODS

### Chemicals and reagents

The chemicals used were: acetic acid (Merck, Germany), castor oil (Sigma Chemicals, USA), diclofenac sodium and loperamide (Square Pharmaceuticals Ltd; Dhaka, Bangladesh), normal saline solution (0.9% NaCl; Orion Infusion Ltd, Bangladesh). Dimethylsulfoxide and Tween-80 were from Sigma–Aldrich and rests of the chemicals used were of BDH and E-Merck analytical grade.

### Preparation of plant sample

Leaves of *Clerodendrum indicum* were collected from Modhupur, Tangail, Bangladesh, in November 2009 and authentification of the sample was confirmed by the taxonomist of the National Herbarium of Bangladesh, Mirpur, Dhaka. A voucher specimen no. has been deposited (accession No: DACB 34556) in the Herbarium for further reference. The leaves were sun dried for several days. After complete drying, the dried leaves were then ground to a coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka. The coarse powder was then stored in an air-tight container marked for identification and kept in a cool, dark and dry place for future use.

### Extraction and partitioning of the plant material and sample preparation

About 900 gm of powdered leave material was taken in a clean, round bottomed flask (5 liters) and macerated at room temperature in 3 liters of methanol for 10 days with occasional shaking for better extraction. The whole mixture was then filtered through cotton followed by Whatman's No. 1 filter paper. After filtration, the filtrate was concentrated at 40 °C with a Heidolph rotary evaporator. The concentrated extract was then air dried to a solid residue. The weight of the crude methanolic extract of leaves obtained was 56 gm. Fractionation of the methanolic extracts was carried out by using solvent-solvent partitioning using the protocol designed by Kupchan[8] and modified version by Wagenen et al.[9] The crude extract (35 gm) was dissolved in 10% aqueous methanol which was subsequently extracted with petroleum ether, carbon tetrachloride and chloroform. All the three partitioning (pet ether fraction, carbontetrachloride fraction and chloroform fraction) fractions were evaporated to dryness by using rotary evaporator and kept in airtight containers for further analysis. The extracts and standard drug (diclofenac sodium, loperamide) were suspended in normal saline using 0.1% Tween-80.

### Experimental animals

Swiss-albino mice (*Mus musculus*) of either sex, aged 4-5 weeks, obtained from the Animal Resource Branch of the International Center for Diarrheal Diseases and Research, Bangladesh (ICDDR, B) were used for the experiment. They were housed in polypropylene cages (30x20x13 cm) and kept in standard environmental conditions (temperature 23 ± 2 °C, relative humidity 55 ± 10% and 12 hours light/dark cycle). The animals were fed with standard rat food (ICDDR, B formulated) and water *ad libitum*. As these animals are very sensitive to environmental changes, they were kept in the environment where the experiment would take place 7 days before the test. The design and performance of research study involving mice was approved by the Ethical Review Committee, Faculty of Biological Science, University of Dhaka through the submission of a research protocol before the study.

### Experimental procedures

#### *Acetic acid-induced writhing response in mice*

The methanolic crude extract and the different fractions of the methanolic extract of the leaves of *Clerodendrum indicum* were subjected to a screening for analgesic activity by acetic acid-induced writhing inhibition method.[10] Initially, the Swiss albino mice were divided into five groups (n=5). Subsequently, vehicle (1% Tween-80 solution in normal saline, 10 ml/kg, as control group), diclofenac sodium (50 mg/kg, as standard), methanolic crude extract (200 and 400 mg/kg) and $CCl_4$ fraction of methanolic extract (200 mg/kg) were administered orally by means of a long needle with a ball-shaped end. After 40 minutes, acetic acid (0.7%, 0.1 mL/10 g) was administered intra-peritoneally to each of the animals of all the groups to induce pain. A forty-minute interval between the oral administration

of test materials and intra-peritoneal administration of acetic acid was given to assure proper absorption of the administered samples. Five minutes after the administration of acetic acid, the number of squirms or writhing characterized by contraction of the abdominal musculature together with turning of trunk and extension of hind limbs, were counted for each mouse for fifteen minutes. Percentage inhibition of writhing in comparison to control group was taken as an index of analgesia and was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{[(\text{Wc-Wt}) \times 100]}{\text{Wc}}$$

Where Wc is the average number of writhing reflex in the control group and Wt is the average number of writhing in the test groups.

### *In vitro* Antimicrobial Screening

The *in vitro* antimicrobial activities of methanolic crude extracts and its different fractions from *Clerodendrum indicum* leaves were examined by the disc diffusion method.[11-14] The bacterial and fungal strains used for the experiment were collected as pure culture from the Institute of Nutrition and Food Sciences (INFS), University of Dhaka. A measured amount of each test sample (methanolic crude extract, pet-ether fraction, chloroform fraction and $CCl_4$ fraction) was dissolved in a specific volume of the solvent (chloroform) to obtain the desired concentrations and applied to sterile discs (6 mm diameter) at a concentration of 400 µg/disc followed by drying off the solvent in an aseptic hood. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic Kanamycin (30 µg/disc) discs and blank discs (impregnated with 10 µl of solvents) were used as positive and negative controls, respectively. These plates were then kept at a low temperature (4 °C) for 24 hours to allow maximum diffusion. During this time dried discs absorbed water from the surrounding media and then the test materials were dissolved and diffused out of the sample disc. The plates were then incubated at 37 °C for 24 hours to allow maximum growth of the organisms. If the test materials have any antimicrobial activity, it will inhibit the growth of the microorganisms and a clear, distinct zone of inhibition will be visualized surrounding the medium. The antimicrobial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment was carried out in duplicate.

### Effect of extract on castor oil-induced diarrhea

The effect of the methanolic crude extract and its different solvent-soluble fractions on castor oil-induced diarrhea was evaluated by the method described by Uddin et al.[15] and Awouters et al.[16] In this method castor oil is used to induce diarrhea in all the experimental groups. The defecation is the primer to measure the anti-diarrheal effect. Thirty experimental mice were randomly selected and divided into six groups consisting of 5 mice in each group. Group I received vehicle (1% Tween-80 solution in normal saline, 10 ml/kg, as control group) and Group II received the standard anti-diarrheal agent loperamide (50 mg/kg p.o.). The third, fourth, fifth and sixth groups were the test groups and received methanolic crude extract and pet ether, $CCl_4$ and chloroform fractions of methanolic extract, respectively at a dose of 400 mg/kg body weight p.o. Prior to any treatment, each mouse was weighed properly and the doses of the test samples and control materials were adjusted accordingly. Each animal was then given 0.5 ml of castor oil orally after 30 min of treatment and placed in transparent cages to observe for consistency of fecal matter and frequency of defecation for 3 h. Feces were collected with an absorbent sheet of paper placed beneath the transparent cages.[17] The wet feces were read at the end of the experiment by lifting up the upper part of the cage containing the sheet of paper and animals. The percent (%) inhibition of defecation was measured using the following formula.

$$\text{\% Inhibition of defecation} = \left[\frac{(A - B)}{A}\right] \times 100$$

A = Mean number of defecation caused by castor oil

B = Mean number of defecation caused by drug or extract

### Statistical analysis

The results obtained were expressed as mean ± SEM. The data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's *t* test to determine the level of significance. A value of $P<0.05$ was considered to be significant. The statistical analysis was carried out using the SPSS program (version 17).

## RESULTS

### Acetic acid-induced writhing response

In the acetic acid-induced writhing test, the methanolic crude extract of leaves of *C. indicum* at a dose of 200 and 400 mg/kg body weight showed a significant ($p<0.001$) and dose-dependent reduction in the number of writhes with 62.57% and 70.76% of inhibition, respectively (Table 1) when compared to the control which were even higher than that of the standard drug diclofenac sodium (55.56% inhibition; $p<0.001$). The carbon tetrachloride fraction of the crude extract at a dose of 400 mg/kg also showed potent anti-nociceptive activity ($p<0.001$) with 73.09% of inhibition of writhing response when compared to control.

### *In vitro* antimicrobial screening

The zones of inhibition produced by methanolic crude extract, carbon-tetrechloride fraction and chloroform fraction of the methanolic extract of *Clerodendrum indicum* (leaves) ranged from 9-13 mm, 10-13 mm and 10-13 mm, respectively at a concentration of 400 µg/disc (Table 2). The methanolic crude extract, carbon-tetrechloride fraction and chloroform fraction showed moderate activity against the tested organisms in terms of both zone of inhibition and spectrum of activity against some gram-positive, gram-negative bacteria and fungi. But the pet ether fraction showed no antimicrobial activity.

**Table 1: Effects of methanolic crude extract and its $CCl_4$ fraction of leaves of *C. indicum* on acetic acid-induced writhing response in mice**

| Treatment | Dose (mg/kg) | Writhing[a] | % inhibition |
|---|---|---|---|
| Control (Vehicle) | 10 mL/kg | 34.2 ± 2.81 | - |
| Diclofenac sodium | 50 | 15.2 ± 1.93* | 55.56 |
| MCE | 200 | 12.8 ± 1.43* | 62.57 |
| MCE | 400 | 10 ± 1.30* | 70.76 |
| CTF | 200 | 9.2 ± 1.48* | 73.09 |

[a]values represent mean ± SEM (n=5). One-way ANOVA followed by Dunnett's t test; *p<0.001: significantly different from control.
MCE: methanolic crude extract of leaves of *C. indicum*; CTF: Carbon-tetrachloride fraction of methanolic extract of leaves of the plant.

### Screening of antidiarrheal activity

The chloroform fraction of the crude extract exhibited significant anti-diarrheal activity, while the methanolic crude extract exhibited moderate anti-diarrheal activity (Table 3). The percent of inhibition of defecation produced by the chloroform fraction of the crude extract was 26.96%, while methanolic crude extract showed 21.74% inhibition at a concentration of 400 mg/kg body weight. The results were found to be comparable to that of standard drug loperamide

**Table 2: Antimicrobial activity of test samples of *Clerodendrum indicum* (leaves)**

| Test microorganisms | Diameter of zone of inhibition (mm) | | | | |
|---|---|---|---|---|---|
| | MCE | CTF | CHF | PEF | Kanamycin |
| **Gram positive bacteria** | | | | | |
| *Bacillus sereus* | 12 | 13 | 10 | - | 27 |
| *Bacillus megaterium* | 10 | 10 | 10 | - | 31 |
| *Bacillus subtilis* | 10 | 10 | 13 | - | 34 |
| *Staphylococcus aureus* | 10 | 13 | 13 | - | 31 |
| *Sarcina lutea* | 10 | 10 | 13 | - | 34 |
| **Test microorganisms** | **Diameter of zone of inhibition (mm)** | | | | |
| | **MCE** | **CTF** | **CHF** | **PEF** | **Kanamycin** |
| **Gram negative bacteria** | | | | | |
| *Escherichia coli* | 13 | 13 | 10 | - | 29 |
| *Pseudomonas aeruginosa* | 10 | 13 | 13 | - | 31 |
| *Salmonella paratyphi* | 10 | 10 | 10 | - | 35 |
| *Salmonella typhi* | 10 | 10 | 10 | - | 28 |
| *Shigella boydii* | 10 | 10 | 10 | - | 31 |
| *Shigella dysenteriae* | 9 | 9 | 10 | - | 27 |
| *Vibrio mimicus* | 9 | 10 | 13 | - | 31 |
| *Vibrio parahemolyticus* | 9 | 10 | 10 | - | 34 |
| **Fungi** | | | | | |
| *Candida albicans* | 10 | 10 | 10 | - | 35 |
| *Aspergillus niger* | 10 | 10 | 10 | - | 34 |
| *Sacharomyces cerevisiae* | 10 | 10 | 13 | - | 34 |

**MCE** = Methanolic crude extract of leaves, **CTF** = Carbon-tetrachloride fraction of crude extract of leaves, **CHF** = Chloroform fraction of crude extract of leaves, **PEF** = Pet-ether fraction of crude extract of leaves. (-): No inhibition.

**Table 3: Effect of leaf extracts of *Clerodendrum indicum* on castor oil-induced diarrhoea**

| Test Sample | Animal Group | Dose mg/Kg | Number of defecation (Mean ± SEM) | % Inhibition of defecation |
|---|---|---|---|---|
| Control | I | 0.2 ml | 115 ± 0.48 | - |
| Loperamide | II | 50 | 72 ± 0.37* | 37.39% |
| Crude extract | III | 400 | 90 ± 0.32* | 21.74% |
| Pet-ether | IV | 400 | 95 ± 0.20* | 17.39% |
| Carbon-tetrachloride | V | 400 | 97 ± 0.79 | 15.65% |
| Chloroform | VI | 400 | 84 ± 1.68* | 26.96% |

Values are expressed as mean ± SEM (n=5); One-way ANOVA followed by Dunnetts t test; *p<0.05 compared to control.

(37.39% inhibition at 50 mg/kg body weight) with regard to the severity of diarrhea. The carbon-tetrachloride soluble fraction and pet-ether soluble fraction of the methanolic crude extract exhibited poor antidiarrheal activity having 15.65% and 17.39% inhibition of defecation, respectively, at a concentration of 400 mg/kg body weight.

## DISCUSSION

The writhing response of the mouse to intraperitoneally injected noxious chemicals such as acetic acid is widely used to screen for peripheral analgesic activity.[18] The intra-peritoneal administration of a 0.7% solution of acetic acid induces endogenous pain mediators, such as prostaglandins, histamine and bradykinin, which stimulate the pain sensation locally.[19] It has been reported that the level of prostanoids, particularly $PGE_2$ and $PGF_{2\alpha}$, as well as lipoxygenase products significantly increased in the peritoneal fluid during writhing test.[20-21] The ability of the extracts to attenuate the acetic acid-induced writhing in mice suggests that they possess analgesic activity. So the observed analgesic activity of the methanolic crude extracts and carbon-tetrachloride fraction of the plant might be due to its possible interference in the biosynthesis, release and/or action of some chemical agents such as prostaglandins and leukotrienes from cyclo-oxygenase and lipo-oxygenase pathway, respectively, which are mainly responsible to block the pain sensation and thereby showed pain-inhibitory activity. Phytochemical study of ethanolic exteracts of leaves of *C. indicum* revealed the presence of alkaloids, steroid, saponin, tannin etc.[6] The presence of steroids, saponins and tannins may be major contributors to the anti-nociceptive activity as previously it has been observed that tannins, saponins and steroidal compounds possess good analgesic activity by inhibiting prostaglandin synthesis.[22-23]

The data from the *in vitro* antimicrobial screening test showed that the methanolic extract and its $CCl_4$ and chloroform partitionates showed moderately potent and broad spectrum antimicrobial activities. It has been documented that saponins, alkaloids and tannins are known to have curative activity against several pathogenic bacteria and fungus.[24] The broad antibacterial activities of the leaf extracts of *C. indicum* might be due to the presence of these compounds (saponins, alkaloids and tannins).

Castor oil-induced diarrheal model is widely used for the evaluation of anti-diarrheal property of drugs. The anti-diarrheal property of the methanolic crude extract and chloroform fraction of methanolic extract of *Clerodendrum indicum* leaves found in the present study could be owing to the presence of tannins, alkaloids, saponins and terpenes in this plant as previous studies have shown that anti-dysenteric and anti-diarrheal properties were mostly due to tannins, alkaloids, saponins, steroids and triterpenes.[25-27]

## CONCLUSION

In conclusion, the results of this study demonstrated that the methanolic extract and different partitioning fractions of leaves of *Clerodendrum indicum* possess significant anti-nociceptive, antimicrobial and anti-diarrheal activities. The findings of the study validated the traditional use of the plant in the treatment of different painful conditions, gastro-intestinal disorders and infectious diseases. Further works are required to isolate and characterize the bioactive compound(s) responsible for the observed analgesic, antimicrobial and anti-diarrheal activities and to evaluate the mechanism(s) of action of these activities.

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# Antibacterial Potential of Gall Extract of *Quercus infectoria* against *Enterococcus faecalis*– an *in vitro* Study

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## ABSTRACT

**Background:** Various herbal products are being tried to treat common ailments. Such a trend is witnessed in dentistry also. Gall extract of *Quercus infectoria* has been found to possess antibacterial properties against some common oral pathogens. **Aim:** To assess the antibacterial property and minimum inhibitory concentration (MIC) of gall extract of *Quercus infectoria* against *Enterococcus faecalis*. **Settings and Design:** *In vitro* experimental study-Laboratory setting. **Materials and Methods:** Dried galls of *Quercus infectoria* were crushed into small pieces and powdered in an electric grinder. About 250 g of the powder was packed into a Soxhlet apparatus and extracted using methanol as the solvent. The crude extract was dried and stored in sterile bottle. The antibacterial property of gall exract of *Quercus infectoria* against *Enterococcus faecalis* was assessed by the disc diffusion method. The zone of inhibition was measured using vernier calipers. The minimum inhibitory concentration of gall extract was assessed by a two-fold serial dilution method. Sodium hypochlorite (2%) and Chlorhexidine (2%) were used as positive controls and dimethyl sulphoxide was used as the negative control in the study. **Results:** The zone of inhibition of gall extract against *Enterococcus faecalis* was found to increase with increasing volumes of gall extract. The minimum inhibitory concentration (MIC) of the gall extract against *Enterococcus faecalis* was 16.6 µl/ml. **Conclusion:** Methanolic extract of galls of *Quercus infectoria* was found to possess antibacterial property against *Enterococcus faecalis*.

**Key words:** disc diffusion method, *Enterococcus faecalis,* methanolic gall extract, *Quercus infectoria,* tannins.

## INTRODUCTION

Dental caries is a complex multi-factorial disease of ubiquitous distribution. It is a major public health problem because it has functional, esthetic, and economic consequences on the individual as well as to the community at large. If left untreated at the earlier stages, dental caries encroaches upon the dental pulp and in such cases endodontic therapy might be the only scope to save the tooth. Endodontic therapy is essentially a debridement procedure that aims at eradication of microbes and their byproducts from the root-canal system. Complex root canal anatomy demands the use of antimicrobial agent in the form of endodontic irrigant along with mechanical debridement to adequately prepare the root canal system.[1]

Microorganisms have been implicated in the development and protraction of pulpal and periapical pathoses of dental caries.[2] Bacterial interactions, poor nutrient availability, and low oxygen potential within root canals with necrotic pulp restrict the number of bacterial species present in endodontic infections.[3] These selective conditions favour the predominance of facultative and strictly anaerobic microorganisms. *Enterococcus faecalis* is one such microorganism known to survive and multiply in the hostile conditions prevailing within the root canal.[4] It is implicated in endodontic failure cases and is said to be more resistant to endodontic treatment.

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There is no single endodontic irrigant that alone sufficiently provides all the functions required for an ideal irrigant.[5] Though 5.25% sodium hypochlorite solution and 2% chlorhexidine are commonly used endodontic irrigants, their usage is not without disadvantages. Sodium hypochlorite is cytotoxic and can cause ulceration, allergic reaction, disagreeable odour and taste.[6] Chlorhexidine on the other hand, has no tissue dissolving property and its activity is reduced in the presence of organic matter.[5] Another area of growing concern is increase in the number of antibiotic resistant strains owing to the irrational usage of synthetic drugs. All these constraints led to a search for herbal alternatives as endodontic irrigants.

*Quercus infectoria* (commonly known as Gall oak) is a small shrub found in Greece, Asia Minor and Iran. The gall arising in the branches of the tree is called as '*majuphal*' in Sanskrit and *'machakai'* in Kannada (both are local languages in India). In India, the galls of *Quercus infectoria* are used since ages as a home remedy for sore throat and chronic diarrhea in both rural and urban areas. It is also used as an ingredient in Ayurvedic preparations.[7] Gall extract of *Quercus infectoria* has shown promising antibacterial potential against some common oral pathogens like *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus sanguis Staphylococcus aureus*, and *Lactobacillus acidophilus*.[8] An *in vitro* experimental study was designed to assess the antibacterial potential and MIC of gall extract of *Quercus infectoria* against *Enterococcus faecalis*.

## MATERIALS AND METHODS

### Preparation of methanolic extract of galls of Quercus infectoria

Dried galls of *Quercus infectoria* were purchased from the local market and identified by a botanist based on its physical characteristics. About 500 grams of the galls were crushed into small pieces in a mortar and pestle and then powdered in an electric grinder. Two hundred and fifty grams of the gall powder was accurately weighed in an electronic balance, packed into a Soxhlet apparatus and extracted exhaustively using methanol as the solvent. The obtained crude extract was evaporated at room temperature to get the dried residue of methanolic extract of galls of *Quercus infectoria*. It was stored in a sterile bottle and preserved in desiccator until further use. A stock solution was prepared by dissolving 10 grams of the extract in 50 ml of dimethyl sulphoxide (DMSO) to obtain a final concentration of 200 mg/ml. For 250 grams of gall powder dissolved in one liter of methanol, 135 grams of the extract (residue) was obtained and the yield was 54% w/w.

### Microbiological procedures

The antibacterial potential of methanolic extract of galls of *Quercus infectoria* was assessed against *Enterococcus faecalis* (ATCC 35550) by agar diffusion method.[9] Brain heart infusion agar media was employed to culture *Enterococcus faecalis*. The concentration of the organism was adjusted to $1.5 \times 10^8$ colony forming units per ml by using 0.5 McFarland Standards and was applied on the surface of the plate. Five wells of 8 mm diameter each were cut in the agar plate and were filled with 75 µl, 50 µl, 25 µl, 10 µl, 5 µl of the gall extract respectively. The agar plates were incubated overnight at 37 °C. The antibacterial activity was interpreted from the size (diameter) of inhibition zone measured in millimeters observed as a clear zone surrounding each well on the agar plate. The zone of inhibition was measured using a a vernier caliper.

Two-fold serial dilution method was employed to find out the MIC of the methanolic extract of galls of *Quercus infectoria* starting from a concentration of 500 µl/ml to 1 µl/ml.[9] A standard amount ($1.5 \times 10^8$ colony forming units/ml) of *Enterococus feacalis* culture was added to the diluted extract in sterile tubes and was incubated overnight at 37 °C. The result was interpreted as sensitive if the supernatant was clear and as resistant if the suspension was turbid. The last tube in the series showing clear supernatant was considered to be the MIC value of gall extract of *Quercus infectoria* against *Enterococcus faecalis*.

### Control agents

Two percent sodium hypochlorite solution and 2% chlorhexidine solution were chosen as the positive control while dimethyl sulphoxide (DMSO), which was used as the solvent for dissolving the methanolic extract of galls of *Quercus infectoria*, was selected as the negative control. The antimicrobial potential of the control agents were tested against *Enterococcus faecalis* in a separate agar plate and were then compared with that of the test agent.

## RESULTS

The data obtained was appraised observationally. Table 1 shows the zone of inhibition for different volumes of the gall extract of *Quercus infectoria* against *Enterococcus faecalis* as compared to that of the control agents. The zone of inhibition was 24 mm when 75 µl of gall extract was used while it decreased to 14 mm for 10 µl. Thus, zone of inhibition of gall extract of *Quercus infectoria* against *Enterococcus faecalis* was found to increase with an increase in the volume of the gall extract tested (Figure 1).

The two fold serial dilution method to assess the MIC showed that concentrations of 500 µl/ml, 250 µl/ml, 125 µl/ml, 62.5 µl/ml, 31.25 µl/ml and 16.6 µl/ml of gall extract of *Quercus infectoria* were found to inhibit bacterial growth while concentrations of 8.4 µl/ml, 4.2 µl/ml, 2.1 µl/ml

and 1.1 µl/ml were found to not inhibit *Enterococcus faecalis*. Thus, the minimum inhibitory concentration of methanolic extract of galls of *Quercus infectoria* against *Enterococcus faecalis* is 16.6 µl/ml (Figure 2).

**Table 1: Zone of inhibition (in mm) for different volumes of gall extract of *Quercus infectoria* against *Enterococcus faecalis* compared to that of control agents**

| Particulars | Zone of inhibition (in mm) |
|---|---|
| **Gall extract (µl) volume/well** | |
| 10 | 14 |
| 25 | 17 |
| 50 | 21 |
| 75 | 24 |
| **Control agents** | |
| 2% Chlorhexidine | 21 |
| 2% Sodium hypochlorite | 0 |
| Dimethyl sulphoxide | 0 |

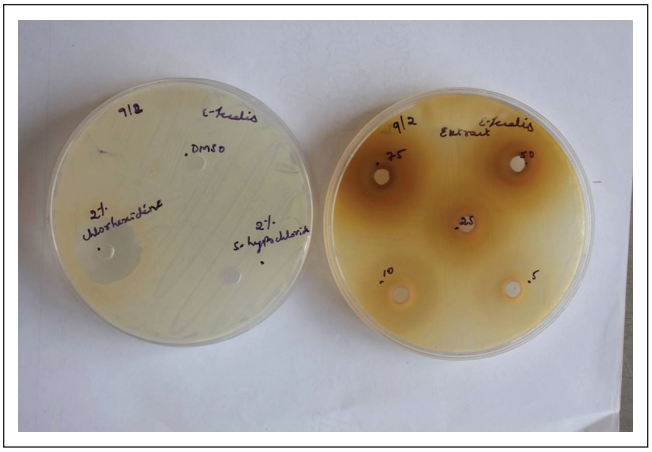

**Figure 1:** Zone of inhibition (in millimeters) for different volumes of test agent compared to positive and negative control against *Enterococcus faecalis*

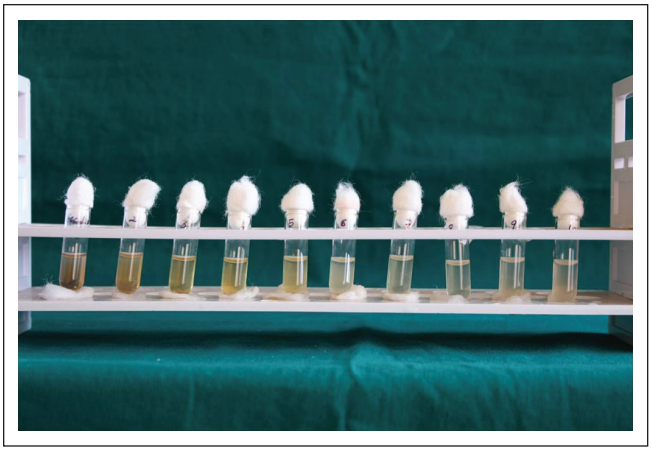

**Figure 2:** Minimum inhibitory concentration (MIC) of methanolic extract of galls of *Quercus infectoria* against *Enterococcus faecalis* by two fold serial dilution method

## DISCUSSION

There is a growing interest to explore the potential of herbal products in treating common ailments. In the field of Endodontics, plant products such as *Morinda citrifolia,* green tea, Triphala, *Arctium lappa, Rosa damascena* have been assessed for its antimicrobial property against endodontic pathogens so that they can be used as an endodontic irrigant.[10-13] Gall extract of *Quercus infectoria* was tested against *Enterococcus faecalis* as it was already found to be effective against some common oral pathogens.[8] Moreover it is easily available, culturally acceptable and has relatively low tissue toxicity. The galls of *Quercus infectoria* have been pharmacologically documented to possess astringent, local anaesthetic, antiviral, antibacterial, antifungal, larvicidal and anti-inflammatory properties.[7,14]

The main constituents found in the galls of *Quercus infectoria* are tannins (50-70%) with small amounts of free gallic acid and ellagic acid.[7] Tannins are phenolic compounds that are known to possess antimicrobial property. The antibacterial property of gall extract of *Quercus infectoria* can thus be attributed to the high concentration of tannins present in it. The active agents in galls are soluble in a variety of solvents such as water, alcohol, petroleum ether and acetone. However, methanolic extract of galls was prepared in the present study based on the study conducted by ArchaVermani et al. which revealed that methanolic extract has more consistent antibacterial property against some common oral pathogens.[8] It is probably because various organic compounds present in galls of *Quercus infectoria* can leach more in methanol.

The zone of inhibition of gall extract of *Quercus infectoria* against *Enterococcus faecalis* was found to increase with an increasing volume and was even found to be greater than that of 2% chlorhexidine (positive control). It is also interesting to note that the other positive control, 2% sodium hypochlorite did not inhibit the growth of *Enterococcus faecalis*. This finding may be attributed to the very low concentration of sodium hypochlorite tested in the present study. Further, the antimicrobial property of a drug will be exhibited only when it diffuses through the solid agar medium. However, an *in vitro* study conducted by Gomes et al. revealed that concentrations as low as 0.5% sodium hypochlorite has antimicrobial property *in vitro*.[15] Dimethyl sulphoxide, which was used as a solvent for gall extract, served as the negative control in the present study. It did not inhibit the growth of *Enterococcus faecalis* suggesting that the zone of inhibition seen for gall extract was solely due to the active ingredients present in it and not because of the solvent used.

A direct comparison of the study results could not be made as there are no previous studies to assess the antibacterial property of the gall extract of *Quercus infectoria* against endodontic pathogens. Further *in vitro* studies need to be conducted in

biofilm models to ensure that the gall extract of *Quercus infectoria* has similar antibacterial potential against *Enterococcus faecalis* in biofilm. There exist no known agents that can mimic sodium hypochlorite in its tissue dissolving property. If proved to be effective then, gall extract of *Quercus infectoria* can be looked upon as an alternative to synthetic endodontic irrigants and it will substantially reduce the usage of sodium hypochlorite during cleaning and shaping of root canals.

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# Anti-inflammatory Activity of *Muntingia calabura* Fruits

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## ABSTRACT

*Muntingia calabura*, the sole species in the genus Muntingia, is a flowering plant, that belongs to Elaeocarpaceae family. This is a fast growing fruit tree. It is a pioneer species that thrives in poor soil, able to tolerate acidic and alkaline conditions and drought. The fruits are commonly called Jamaican cherry and are red in colour. The flowers are used as an antiseptic and to treat abdominal cramps and spasms. It is also taken to relieve headaches and colds. *Muntingia calabura* fruits possess antioxidant property. However, their anti-inflammatory activity has not been investigated so far. The aim of this study, therefore, was to evaluate the anti-inflammatory activity from the fruits of *M. calabura*. The methanolic fruit extracts (100, 200 and 300 mg/kg i.p.) reduced the Carrageenan-induced edema of the hind paw of adult male Wistar Albino rats in 3 hours. The activity was compared with that of the standard drug indomethacin. Acute toxicity was investigated and the results indicated no abnormalities in the behaviour and lethality by the extract up to 1000 mg/kg. These results indicate the fruit extract of *M. calabura* possess potent anti-inflammatory activity. Therefore, these pharmacological results clearly support traditional folkloric application of *M. calabura* fruits in the control and/or pain, inflammatory illness as well as an antioxidant agent.

**Key words:** *muntingia calabura* fruits; elaeocarpaceae, anti-inflammatory, antioxidant activity

## INTRODUCTION

Plants are potent biochemical factories and have been components of phytomedicine. Since time immemorial man is able to obtain from them a wondrous assortment of industrial chemicals. Plant-based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc., that is, any part of the plant may contain active components.[1]

The protective effect of fruits and vegetables has generally been attributed to their antioxidant constituents, including vitamin C (ascorbic acid), Vitamin E (tocopherol), carotenoids, glutathione, flavonoids and phenolic acids, as well as other unidentified compounds.[2]

Various herbal medicines derived from plant extracts are being used in the treatment of a wide variety of clinical diseases, though relatively little knowledge about their mechanism or action is known.[3] Many herbal preparations are being prescribed widely for the treatment of inflammatory conditions.[4] There is a need for research and developmental work in herbal medicine because apart from the social and economic benefits, it has become a persistent aspect of present day health care in developing countries.

Plant secondary metabolites have provided an important source of drugs since ancient times and now around half of the practical drugs used are derived from natural sources.[5]

Some research has shown that flavonoid compounds are present in various plants; exert beneficial effects on human health such as cardiovascular protection, anti-cancer activity, antinociceptive activity and anti inflammatory effects.[6,7,8]

It is known that there are links between the inflammatory and nociceptive, oxidative and cancer processes. The ability to inhibit any of the processes will definitely lead to the inhibition of the others.[9]

Water soluble extract from leaves of *M. calabura* produced potent antinociceptive and anti-inflammatory activities. The preliminary phytochemical analysis performed in *M. calabura* leaves showed the presence of flavonoids, chalcones, terpenoids and phenolic compounds. The constituents responsible for the analgesic and anti inflammatory effects of *M. calabura* have not yet been elucidated.[10]

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It was scientifically proved that *M. calabura* leaves possess anti-inflammatory and antipyretic activity.[11] Plant secondary metabolites have provided an important source of drugs from ancient times and now around half of the practical drugs used are derived from natural sources.[5]

## MATERIALS AND METHODS

### Plant materials

*Muntingia calabura* plant belongs to the family Elaeocarpaceae. The fruits of *M. calabura* were collected from the surrounding areas of Erode district, Tamilnadu, India and the plant was identified, authenticated and deposited (Voucher number:BSI/SC/5/23/09-10/Tech-132) at Tamil Nadu Agricultural University, Coimbatore, Tamilnadu.

### Preparation of methanolic extract

The fruits of *M. calabura* were freshly collected and extracted with methanol; the extract was completely dried in vacuum, stored in refrigerator at 4ºC and protected from sunlight for further use.

### Antioxidant activity

#### *DPPH radical scavenging assay*

The effect of fruit extracts on DPPH (1,1-diphenyl picracyl hydrazine) radical was determined.[12] Different concentrations of the extracts (500, 400, 300, 200, 100 µg/ml) were prepared and subjected to antioxidant tests. To 1 ml of each of the extracts, 5 ml of 0.1mM methanol solution of DPPH was added, vortexes, followed by incubation at 27 °C for 20 min. The control was prepared without any extract and absorbance of the sample was measured at 517 nm using UV/VIS Spectrophotometer (ELICO) using methanol to set 0. The ability to scavenge DPPH radical was calculated by the following equation:

$$\% \text{ inhibition} = 100 \times \frac{Abs_{control} - Abs_{sample}}{Abs_{control}}$$

### Pharmacological tests

#### *General animal preparation*

Experiments were performed on healthy male Wistar Albino rats (120-150 g), procured from the small animals breeding station, Mannuthy, Kerala, India. They were housed in polypropylene cages (38 × 23 × 10 cm) with not more than six animals per cage and maintained at standard environmental conditions (14 /10 hrs dar/light cycles; temp 25 ± 2 °C; 35-60% humidity, air ventilation) and were fed with standard pellet diet (M/s Hindustan Lever Ltd., Mumbai, India) and fresh water *ad labium*. The rats were acclimatized to the environment for two weeks prior to experimental use. Animals were fasted overnight before the experimental schedule, but had free access to water *ad libitum*. All animal procedures were performed in accordance with Institutional Animal Ethic Committee (IAEC) guidelines, after getting the approval of the committee for the purpose of control and supervision of experiments on animals (CPCSEA) in Karpagam University, Coimbatore. (CPCSEA No: 739/03/abc/CPCSEA).

Adult male Wister rats taken for the study were divided into 6 groups; each group containing 6 animals and each group was treated as follows:

| Groups | Treatment |
|---|---|
| Group 1 | Control rats (normal saline only) |
| Group 2 | Inflammation by carageenan |
| Group 3 | Standard drug |
| Group 4 | 100 mg/kg fruit extract + carageenan |
| Group 5 | 200 mg/kg fruit extract + carageenan |
| Group 6 | 300 mg/kg fruit extract + carageenan |

### Preparation of test agents

The methanol extract of *M. calabura* fruit was dissolved in isotonic normal saline (0.9% w/v) to make a stock solution with a concentration of 100 mg/ml. Three different doses at 100, 200, 300 mg/kg were injected into the animals. Additional test agents used in this study included Carrageenan and indomethacin. All chemicals and the extract were administered orally. All drugs and the extract were freshly prepared before use and dissolved in isotonic normal saline (0.9% w/v), which served as the vehicle and volume control for all agents. The only exception was Carrageenan, which was 0.5% CMC in distilled water served as the vehicle control.

### Anti-inflammatory activity

The anti-inflammatory activity of the fruit sample was investigated in Carrageenan-induced inflammatory model. Acute inflammation was induced in rats.[13] The control group was administered with the saline solution only, while the third group was treated with indomethacin (10 mg/kg p.o.). The fourth, fifth and sixth groups were administered with the fruit extract (100, 200 and 300 mg/kg/day p.o.) respectively. One hour after the administration of fruit extract, the standard indomethacin acute inflammation was produced. Acute inflammatory edema was induced by subplantar injection of 0.1 ml 1% Carrageenan in the right hind paw of each rat in all the groups except the control group. The thickness (mm) of the paw was measured immediately and at 30 mins interval for four hrs after the Carrageenan injection, by using vernier calliper.[14] The percentage of inhibition of edema was calculated for each dose using the following formula:

$$\% \text{ Inhibition} = \left(\frac{1 - PV_T}{PV_C}\right) \times 100$$

$PV_T$ = Paw volume in drug treated group of rats

$PV_C$ = Paw volume in control group of rats

### Statistical analysis

All the values were expressed as mean ± SD. The data were assessed using one way analysis of variance (ANOVA) followed by student's t' test. Statistical significance was accepted at $p<0.05$.

## RESULTS

In our study, the fruits of *M. calabura* were evaluated for its antioxidant activity using DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. The $IC_{50}$ value was obtained for the tested assay, which showed that the lower the $IC_{50}$ value, the higher was the antioxidant activity (Table 1).

Percentage inhibition of edema, volume of methanol and standard drugs were calculated after every hour for five hours. There was a significant and dose dependent anti-inflammatory activity of methanolic extract in the Carrageenan-induced rat paw edema model. Results of the effect of *M. calabura* fruit extract in Carrageenan-induced edema in test rats are shown in Figure 1 and Plate 1. Edema was greatly suppressed irrespective of the dose level of extract used and was comparable to the standard indomethacin treatment.

The methanol extract of *M. calabura* fruit at the dose levels of 100, 200 and 300 mg/kg caused a dose dependent inhibition of localized swelling caused by Carrageenan at 4 hrs (Table 2). The significant anti-inflammatory effect was dose dependent with 24.36% reduction observed for 100 mg/kg and 44.14% seen for 200 mg/kg dose and 62.43% observed for 300 mg/kg dose. Further, the protection induced by 300 mg/kg was also found to be as potent as indomethacin (80.48%) in reducing paw edema.

## DISCUSSION

The fruit extract demonstrated H-donor activity. With regard to the estimated $IC_{50}$ value, the extracts of *M. calabura* displayed significant DPPH radical quenching property. The DPPH assay constitutes a quick and low cost method, which has frequently been used for the evaluation of the antioxidant property of various natural products.[1]

Herbal products are consumed in traditional medical systems as functional/recreational food supplements or as medicines in many countries. In recent years, evidence has accumulated to suggest that complementary medicine for treatment of

AQ:1 **Table 1: The percentage inhibition on DPPH radical by *M. calabura* fruit extract**

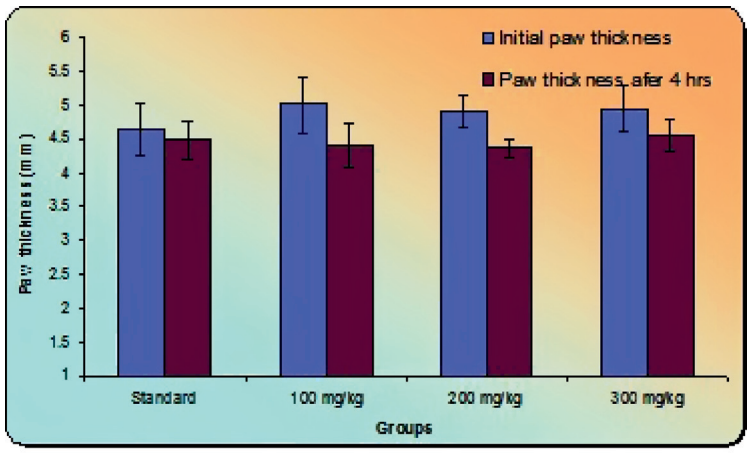


**Figure 1:** The anti-inflammatory effect of *M. calabura* fruit extract on paw edema in Wistar Albino rats

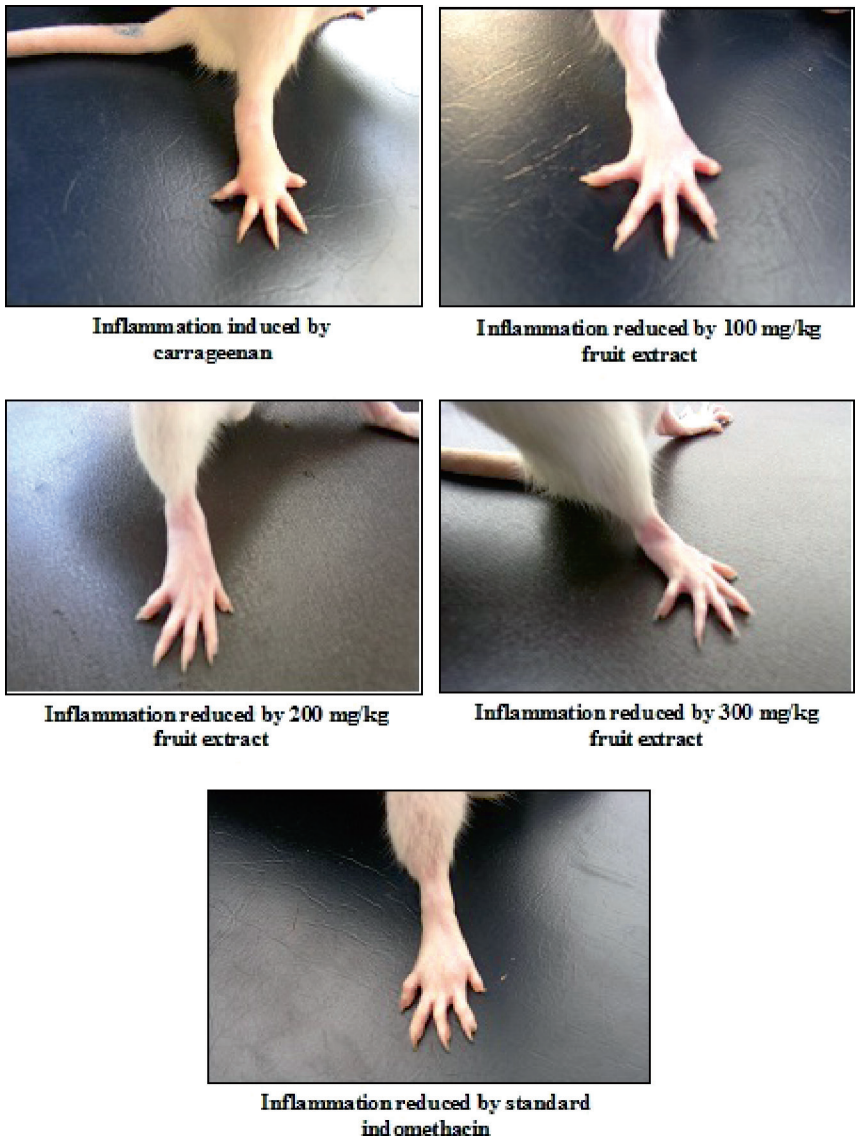


**Plate 1:** The anti-inflamatory effect of *M. calabura* fruit extract on Carrageenan-induced Wistar Albino rats

**Table 2: The effect of fruit extract on percentage inhibition of paw volume**

AQ:2

various diseases is another more popular choice.[15] Many plant extracts of botanical medicinal herbs have been shown to relieve disease's symptoms comparable to those obtained from allopathic medicines. Furthermore, chemical therapeutics are often associated with severe adverse effects. Therefore, safer compounds of natural products with fewer side effects are needed. In this study, demonstrations have produced novel observations for the first time that the fruit extracts of *M. calabura* possess anti-inflammatory effects in Carrageenan-induced hind paw acute inflammation.

In most instances, however the effects of the extracts were significant and dose dependent. The observed anti-inflammatory effects of *M. calabura* fruit extracts could be due to the presence of biologically active chemical constituents in the extracts.

*M. calabura* has been used widely in both tropical America and Southeast Asia.[16,17]. In East Asia, flowers are used for the treatment of headaches and incipient cold or as tranquillizers, anti-spasmodics and antidyspeptics. Recently, ethyl acetate soluble extracts from the leaves of *M. calabura* and its major constituent, flavonoids, have been reported to have chemopreventive effects.[18] In order to evaluate anti-inflammatory effects of *M. calabura* fruit extract on the acute inflammation process, the rat paw edema model was used. In this experimental model, the Carrageenan-induced edema at 4 hrs was significantly inhibited by the fruit extract. The significant anti-inflammatory effect was dose dependent. This data supports the hypothesis of the effect of *M. calabura* fruit on the inflammation mediators in inflammatory processes. It is evident that Carrageenan is a sulphated mucopolysaccharide obtained from the sea weed (*Rhodophyceae*), perhaps the most commonly used to induce acute inflammation producing a maximal edematous in 3 to 5 hrs.[19] While the Carrageenan-induced edema model is typically associated with the activation of the cyclo-oxygenase pathway and is a multi-mediated phenomenon with the release of various inflammation mediators.

The inhibition of Carrageenan-induced inflammation in rats is an established model for evaluating anti-inflammatory drugs, which has been used frequently to access anti-edematous effect of nature products. Similar results were obtained from the aqueous fruit pulp extract of *Hunteria umbellate*[20] and *Ammomum subulatum* fruit extract.[21]

One of the mechanisms that could be used to explain the association between the anti-inflammatory and anti-oxidant activities is the reaction caused by ROS (Reactive oxygen species). ROS which is a type of inflammatory stimulus, has been shown to cause the release of nitric oxide (NO), a compound known to modulate a great number of physiological functions including the peripheral and central nociceptive processing within the body[22,23] It is suggested that the blocking of ROS will cause a decrease in NO synthesis, which in turn will lead to the anti-inflammatory, anticancer and anti-oxidant activities.[24,25]

The edema induced in the rat paw by the injection of 1% Carrageenan is brought about by autocoids, histamine and 5-hydreoxytyptamine (5-HT) during the first one hour, after which kinnins act, to increase the vascular permeability upto two and a half hours. The maximum inflammation is seen approximately three hours post the Carrageenan injection, after which it begins to decline. Following that the prostaglandins act from two and a half hours to six hours, which results in the migration of leucocytes into the inflamed site.[26,27] The pharmacological properties of safflower have been evaluated for antitumor, sedative,[28] antimicrobial, [29] anti-inflammatory and analgesic effects.[30]

*M. calabura* shows a significant inhibition of inflammation, which is comparable to the standard drug indomethacin. In summary, our results demonstrated that the fruit extracts of *M. calabura* possess antioxidant activity and anti-inflammatory activities, similar to those observed for non-steroid drugs such as indomethacin. These findings provide scientific supporting evidence for the therapeutic uses of *M. calabura* fruits in folk medicine. Further studies are required to identify the actual chemical components that are present in the crude extracts of this plant which are responsible for anti-inflammatory activity.

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