

Phytochemical Investigation and Antimicrobial Activity Testing of *Phyllanthus fraternus* Leaves Webster

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Available online: 1st September 2014

ABSTRACT

The present study aimed at the phytochemical evaluation and in vitro antimicrobial effect of extracts from *Phyllanthus fraternus* Webster. on bacteria (*Escherichia coli* and *Staphylococcus aureus*). Extraction method used was maceration. Three solvents were used for extraction of phytoconstituents (pet ether, methanol and hydromethanolic). Only steroids were found in pet ether extract while significant presence of steroids, glycosides, alkaloids, saponins, triterpenoids was found in methanolic extract. Hydromethanolic fraction also showed presence of steroids, glycosides and alkaloids. The fractions of methanolic extract of *Phyllanthus fraternus* were isolated by column chromatography by using solvent system in which polarity was increased gradually. Antimicrobial activity of all the fractions from column chromatography was then checked by agar well diffusion method which shown inhibitory effect for methanolic extract.

Keywords: *Phyllanthus fraternus* Webster, phytochemical investigation, antimicrobial activity

INTRODUCTION

Medicinal plants are the nature's gift to human being to make disease free healthy life. Herbal medicine is still the mainstay of about 75-80 % of the whole population, mainly in developing countries, for primary health care because, better compatibility with the human body and fewer side effects. In India, thousands of species are known to have medicinal values and the use of different parts of several medicinal plants to cure specific ailments has been in vogue since ancient times. From over 3, 00,000 species of higher plants to occur in nature, only about 2% have been screening so far. Extract of plants from 157 families have been reported to be active against microorganisms.¹

BHUIAMLA (*Phyllanthus fraternus* Webster) is an annual herb commonly occurring in gardens, waste places and roadside. In Ghanaian traditional medicine, the leaves of the plant have been used extensively for many years in the treatment of broad spectrum of diseases. This plant belongs to Euphorbiaceae family. In India it is used as an herbal medicine and called 'Bhumyamlaki'. It is a large genus comprising about 750 species in tropical and subtropical region. *Phyllanthus amarus*, a member of the same family was studied for its phytochemical analysis and it was shown that it contains lignan niranthin, nirtetralin and phyltetralin and other compounds like alkamide, alkaloid, terpenoid, and flavonoid. The common uses of plants are whole plant is used in dyspepsia, vertilago,

malaria, diabetes, menorrhagia, sores, chronic dysentery, tubercular ulcers, wound, bruises, scabies, ringworm, dropsical infection, gonorrhoea, genito-urinary disorders, jaundice, indigestion, intermittent fever, anemia, cough, gout, urinary disease, dermatosis, miscarriage, abdomen tumour, vaginitis and skin eruption. Leaves are used in scabies, bruises, wound, poultice lesions, swelling, ulcer, spleen and liver disorders and problem of joints. Bark is purgative. Stem is used in ophthalmia.^{2, 3} The present study included phytochemical screening of leaf extract of *Phyllanthus fraternus* and evaluating them for antimicrobial activity.

MATERIAL AND METHODS

Plant collection and preparation: Fresh leaves of *Phyllanthus fraternus* were collected from Botanical garden of Indira College of Pharmacy, Wakad, Pune. Their botanical identifications and authentication were confirmed at the Agharkar research institute, Pune. Then the leaves were separated and washed with water and allowed to dry. Later fine ground powder was prepared.

Table 1: Percentage yield of *phyllanthus fraternus* species

Sr. No.	Name of extract	Percentage yield
1	Pet ether	36.7
2	Methanolic	57.87
3	Hydro alcoholic	2.0

Table 2: Results of Phytochemical analysis.

Test	Pet ether extract	Methanolic extract	Hydromethanolic extract
Carbohydrate	-	-	-
Protein	-	-	-
Amino acid	-	-	-
Steroids	+	+	+
Glycoside	-	+	+
Flavonoid	-	-	-
Alkaloid	-	+	+
Tannin	-	+	-
Saponin	-	+	+
Triterpenoids	-	+	-

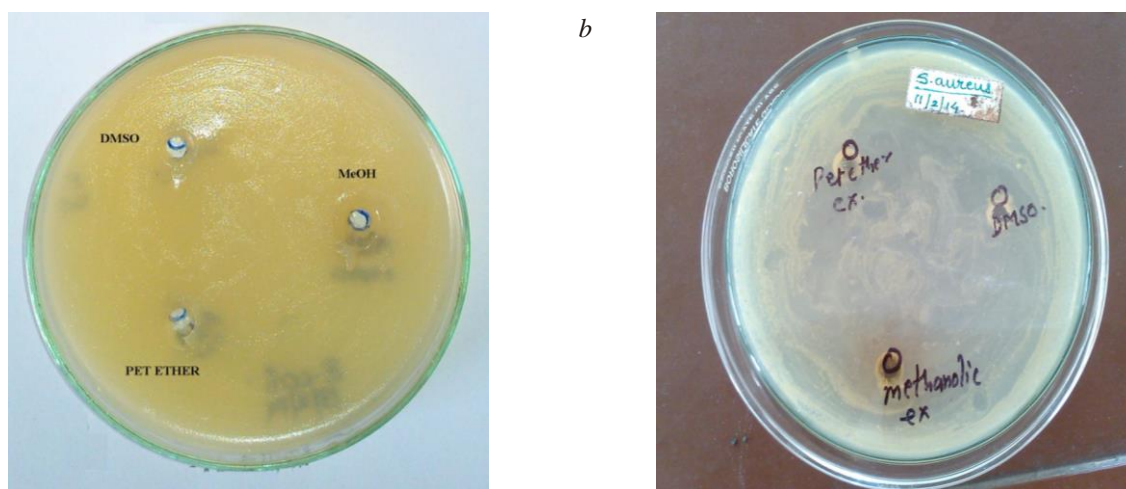


Fig. 1: Zone of inhibition for pet ether and methanolic extract (DMSO as control) on a) *E. coli* and b) *S. aureus*

Crude Extraction (Cold Maceration): 100 g of finely powdered drug was weighed and defatted with 100 ml of pet. ether. The solution was kept overnight with continuous stirring at 350 rpm with the help of magnetic stirrer. Later this solution was filtered through whatmann filter paper and filtrate was concentrated on rotary evaporator to get pet ether extract. The marc obtained was treated with 100 ml of methanol and processed in similar manner as that of pet ether extract to get methanolic extract. Further hydromethanolic extract was obtained by treating marc of methanolic extract with hydromethanolic solution (Methanol: water; 60:40) in similar manner. Percentage yield is given in Table 1.

Phytochemical Analysis of Extracts:^{4,5}

The following procedures were adopted for testing the presence of various chemical constituents in all the three extracts viz. Pet ether, methanolic and hydromethanolic. The results of which are presented in Table 2. Also presence of phytochemicals was further confirmed by using spraying reagents on TLC plate.

Test for Steroids:

Salkowski test: Chloroform (2 ml) and 2 ml of concentrated sulphuric acid were added to 2 ml of test solution, shaken and allowed to stand. Change in the colour of lower chloroform layer to red and acid layer to greenish yellow fluorescence indicates the presence of steroids.

Liebermann-Burchard reaction: T.S 2 ml was mixed with chloroform (2 ml). To the solution, 2 ml of acetic anhydride and 2 drops of conc. Sulphuric acid from the side of test tube were added. Change in colour as, first red, then blue and finally green indicates the presence of steroids.

Test for Triterpenoids:

Salkowski test: Concentrated sulphuric acid (2 ml) was added to 2 ml of test solution. The solution was shaken and allowed to stand. The colour of lower layer changes to yellow indicating the presence of triterpenoids.

Liebermann-Burchardt Test: The 3 ml of test solution was treated with 3 ml of acetic anhydride, mixed well and then 2 ml of concentrated sulfuric acid was added from the sides of the test-tube. The development of deep red colour indicates the presence of triterpenoids.

Test for Glycosides:

Balget's test: 2 ml of the test solution was treated with 2 ml of sodium picrate solution. The development of yellow to orange colour indicates the presence of cardiac glycosides

Keller-Killiani test: Glacial acetic acid (3-5 drops), one drop of 5% FeCl₃ and conc. Sulphuric acid were added to the test tube containing 2 ml of T.S. Appearance of reddish-brown color at the junction of two layers and bluish green in the upper layer indicates the presence of glycosides

Legals test: To 2 ml of test solution, 1 ml of pyridine and

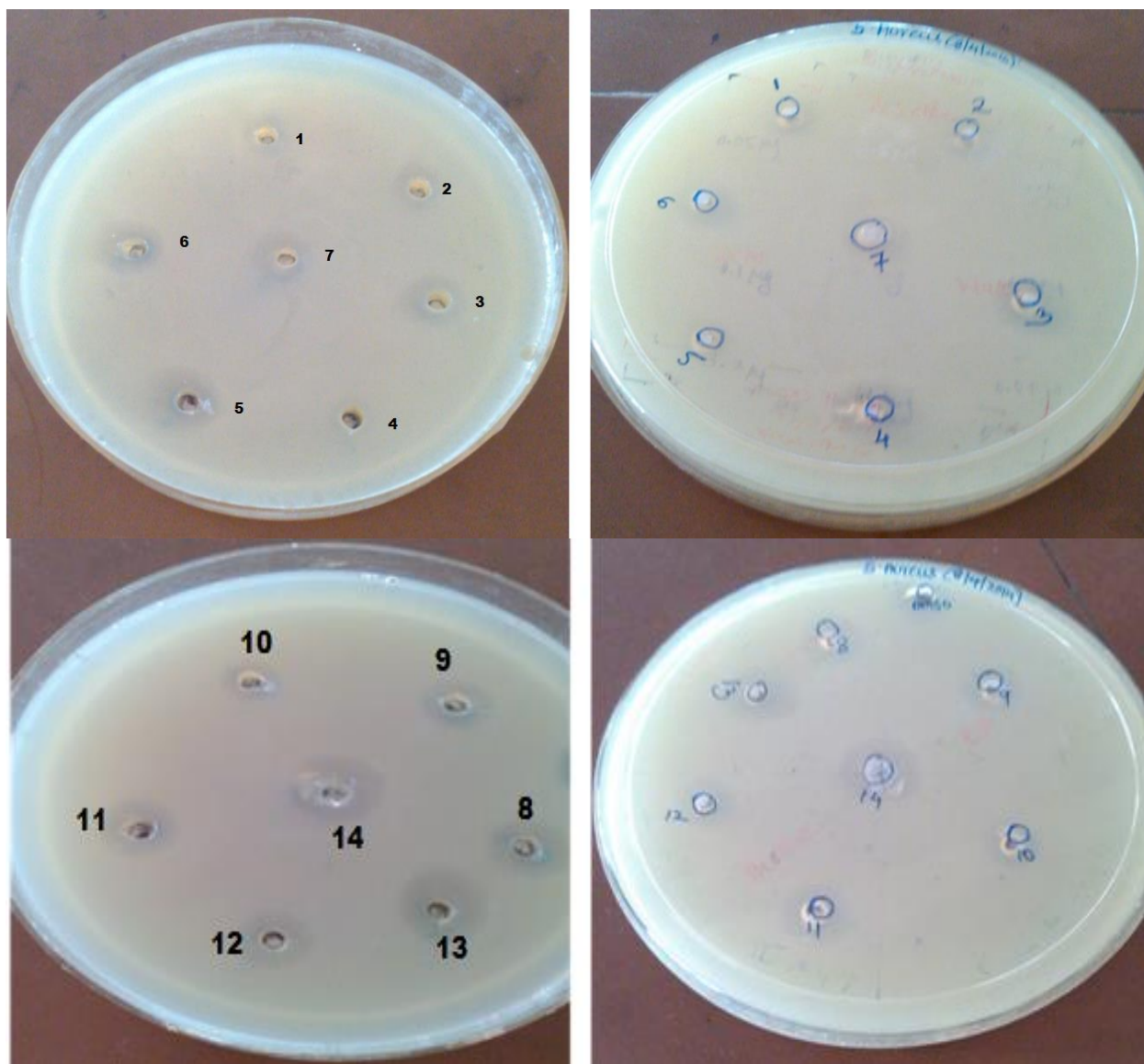


Fig. 2: Zone of inhibition shown by methanolic fractions (Fractions 1-14) on *S. aureus*

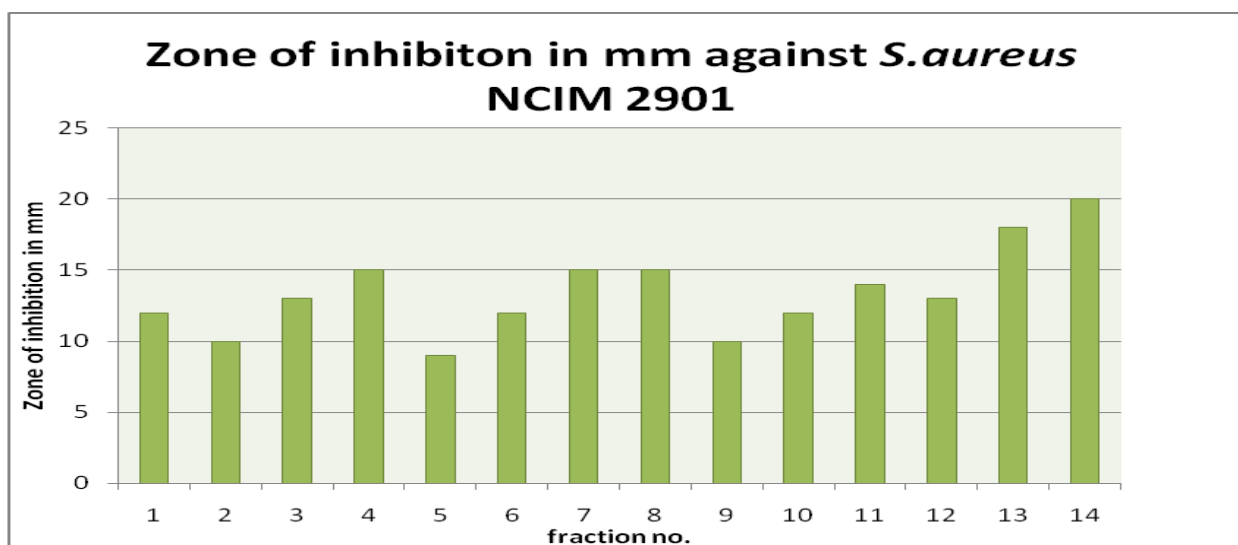


Fig. 3: Plot of Zone of inhibition in mm against fraction no. against *S.aureus* NCIM 2901

1 ml of sodium nitroprusside was added. Change in color to pink or red indicates the presence of cardiac glycosides. Borntrager's test: Dilute Sulphuric acid was added to 2 ml of solution of extract, boiled for a few mins and filtered. To the filtrate 2 ml of benzene or chloroform was added and shaken well. The organic layer was separated and ammonia was added. The change in colour of ammoniacal layer to pink-red indicates the presence of anthraquinone glycosides.

Tests for Saponin:

Foam Test: Powdered extract (10-20 mg) was shaken vigorously with water (1 ml). Development of persistent foam which is stable at least for 15 minutes indicates the presence of saponin.

Tests for Carbohydrates:

Molisch's test: 3 ml of Molisch's reagent was added to the 3 ml of test solution, shaken for few minutes. Then 2 ml of concentrated sulphuric acid was added slowly from the sides of the test tube. The development of a purple ring at the junction of two liquids indicates the presence of carbohydrates.

Barfoed's test: Barfoed's reagent (1 ml) and test solution (1 ml) were mixed in a test tube, heated in boiling water bath for 1-2 min. and then cooled. The appearance of red precipitate indicates the presence of monosaccharides.

Fehling's test: Fehling's A and B solutions (1 ml each) were added to the test tube and boiled for 1 min. To this 2 ml of test solution was added and heated in boiling water bath for 5-10 min. Appearance of yellow and then brick red precipitate indicates the presence of reducing sugars.

Benedict's test: Benedict's reagent (1 ml) and T.S (1 ml) were mixed in a test tube and heated in boiling water bath for 5-10 min. Change in colour to yellow; green or red indicates the presence of reducing sugar.

Tests for Alkaloids: To the dry extract (20 mg) dilute hydrochloric acid (1-2 ml) was added, shaken well and filtered. With filtrate the following tests were performed.

Mayer's test: To the 3 ml of test solution 3 drops of Mayer's reagent (potassium mercuric iodide) was added. Appearance of reddish brown or cream precipitate indicates the presence of alkaloids.

Hager's test: To 3 ml of filtrate 4-5 drops of Hager's reagent (saturated picric acid solution) was added. Appearance of yellow precipitate indicates the presence of alkaloids

Dragendorff's test: 3 ml of the test solution was mixed with Dragendorff's reagent (potassium bismuth iodide). Appearance of reddish brown precipitate indicates the presence of alkaloids.

Tests for Flavonoids:

Ferric-chloride test: Test solution with few drops of ferric chloride solution shows intense green colour indicating the presence of flavonoids.

Shinoda test: To the powdered extract (10 mg), 5 ml of ethanol (95%), 3 drops of hydrochloric acid and 0.5 gm magnesium turnings were added. Change of colour of solution to pink indicates the presence of flavonoids.

Tests for Tannins:

Ferric-chloride test: 3 ml of test solution treated with few drops of ferric chloride solution. Development of dark colour indicates the presence of tannins.

Gelatin test: 3 ml of test solution when treated with gelatin solution (3 ml) gives white precipitate indicating the presence of tannins.

Test for Proteins and Amino Acids:

Millon's test: T.S (3 ml) and Million's reagent (5 ml) were mixed in a test tube. The appearance of white precipitate changing to brick red or dissolved and gave red color to solution on heating indicates the presence of proteins.

Xanthoproteic test: To the test tube containing T.S (3 ml), 1 ml of conc. Sulphuric acid was added. Appearance of white precipitate which turns yellow on boiling and orange on addition of NH_4OH indicates the presence of tyrosin and/or tryptophan containing proteins.

Biuret test: 3 ml of the test solution was treated with 4% sodium hydroxide (3-5 drops) and 1% copper sulphate solution (3-5 drops). The appearance of blue colour indicates the presence of proteins.

Ninhydrin test: Test solution (3 ml) and 3 drops of 5% lead acetate solution were boiled on water bath for 10 min. Change in the colour of solution to purple or blue indicates the presence of amino acids.

Column Chromatography:

- Preparation of sample:

Methanolic extract and dried silica were taken in an appropriate proportion (1:40) and mixed uniformly till free flowing powder of sample is obtained. This sample was used for column chromatography.

- Preparation of column:

Silica gel (60-120 mesh) was dried by keeping it in oven at 100°C for 1 hr. Glass Column was packed by slurry method. In this method slurry of silica gel was prepared with pet ether and it is poured in to the column carefully, care must be taken to avoid air bubbles. Prepared sample was added over the silica.

- Elution of column:

Column was eluted with n-hexane: ethyl acetate: methanol as the mobile phase in increasing proportion of polarity. Every 10 ml of eluent was collected in separate test tubes. Determination of antimicrobial activity: The CLS guidelines were followed for antimicrobial activity⁶. Initially Pet ether extract and methanolic extract (after evaporation of the organic solvents) were tested for antimicrobial activity against Gram negative (*E. coli* NCIM 2133) and gram positive (*S. Aureus* NCIM2901) bacteria. After the initial testing of the extract it was found that zone of inhibition was not found against *E.coli* NCIM 2133, hence it was concluded that both the extract does not show antimicrobial activity on gram negative bacteria. Whereas the growth of *S. aureus* NCIM 2901 was inhibited by methanolic extract. Thus, methanolic extract fractions were further tested for antimicrobial activity on *S.aureus* NCIM 2901.

The fractions obtained from the column chromatography were used for studying their antimicrobial activity. The different collected fractions were dried to powder and then again reconstituted in DMSO solvent (AR grade). DMSO was also checked for antibacterial activity as a control.

Pour plate technique was employed using Muller Hinton Agar (Oxoid) with a count of 10^6 cfu/ml of *S. aureus* NCIM 2901. The experiment was performed under strict aseptic conditions. A well was made in the plates with sterile borer. The fraction was introduced into the well (50 μ l). The fractions were allowed to diffuse by placing the plates at 4°C. The plates were then incubated at 37°C for 24 hrs. All samples were tested in duplicates. Inhibition was determined by measuring the diameter of zone of inhibition using vernier callipers.

RESULTS AND DISCUSSION

The present study shows that percentage yield is highest in methanolic extract. Phytochemical screening showed presence of only steroids in pet ether extract. Significant presence of steroid, alkaloid, glycoside, tannin, saponin and triterpenoids was found in methanolic extract. No effect of extract seen on *E. coli* NCIM 2133 but polar fractions of methanolic extract of leaves of *Phyllanthus fraternus* showed better antibacterial activity against *S. aureus* NCIM 2901 when studied by Agar well diffusion method for MIC determination.

REFERENCES

1. Rajeshwar Y*, Sreekanth T, Shyamsunder A, Tejaswi Divya B, Investigation of Phytoconstituents, TLC Profile and Antimicrobial Activity of Methanol Extract of *Asparagus racemosus* willd. Roots, *International Journal of Pharmacognosy and Phytochemical Research* 2014; 6(1); 128-132
2. Christian M, Steroids – Chemical Constituents of *Phyllanthus Fraternus* Webster through TLC and HPTLC. *International research journal of chemistry*, 39-49
3. Sen B, Dubey SD., Tripathi k., Pharmacognostical Study of Tamalaki (*Phyllanthus Fraternus* Webster) a Herb used Tamaka-svasa, *An international Quaterly journal of research in ayurveda*, 2011;32(3) ; 398-401
4. Mehta K, Patel BN, Jain BK, Phytochemical analysis of leaf extract of *phyllanthus fraternus*, *Research journal of recent science* 2013; 2: 12-15.
5. Lawand R, Gandhi S, comparison of curcuma caesia Roxb with commonly used curcuma species by HPTLC, *journal of pharmacognosy and phytochemistry*, 2013; 2(4); 126-131.
6. Cavaleiri S, Manual of antimicrobial susceptibility testing, ISBN 1-55581-349-6