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PRELIMINARY PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF AQUEOUS EXTRACT OF *Phyllanthus maderaspatensis*

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ABSTRACT

The phytochemical screening and antimicrobial activity of *Phyllanthus maderaspatensis* against a wide variety of pathogenic bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Salmonella typhimurium*, and their efficacies were then compared by the disc diffusion method. Qualitative phytochemical analysis of *P. maderaspatensis* confirms the presence of various phytochemicals like saponins, carbohydrate, proteins, and tannins with various R_f values (0.05, 0.27, 0.40) identified by TLC. At the concentration of 100 mg/ml the *P. maderaspatensis* showed maximum activity (25 mm) against *Staphylococcus aureus*. Others showed moderate activity. The solvent (water) were used as control whereas gentamicin was used as reference for bacteria. The solvent had no effect on the microorganisms whereas gentamicin inhibited microbial growth. The minimum inhibitory concentration (MIC) of the aqueous extracts on *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Salmonella typhimurium* were at 75 mg/ml, 25 mg/ml,25 mg/ml, 75 mg/ml, 50 mg/ml and 25 mg/ml while the minimum bacterial concentration (MBC) were at 75 mg/ml, 50 mg/ml, 25 mg/ml, 75 mg/ml, 50 mg/ml and 25 mg/ml respectively. The observed antibacterial effects were believed to be due to the presence of saponins, carbohydrate, proteins, and tannins identified in the extracts. The results apparently justified their use in the treatment of infections.

Keywords: *Phyllanthus maderaspatensis*, MIC, MBC, Thin layer chromatography (TLC), Antibacterial activity, Phytochemicals.

INTRODUCTION

Microorganisms have developed resistance to many commercial antibiotics due to indiscriminate use of antimicrobial drugs and this has created immense clinical problem in the treatment of infectious diseases. The increase in resistance of microorganism encouraged scientists to search for new natural products from plants for the discovery of new antimicrobial agents as well as an alternative route for the substitution of synthetic chemicals because of their less side effects and the strong resistance towards various microorganisms.[1] Plant based antimicrobial agents are very good source for medicines. They are effective in the treatment of infectious diseases while simultaneously

mitigating many of the side effects that are often associated with synthetic antimicrobials.[2]

Phyllanthus is the largest genus in the family *Phyllanthaceae*. *Phyllanthus* has a remarkable diversity of growth forms including annual and perennial herbaceous, arborescent, climbing, floating aquatic, Pachycauls and phyllocladus. *Phyllanthus maderaspatensis* (*Euphorbiaceae*) is an annual shrub distributed in India, Sri Lanka, China, Africa, Australia, and Indonesia. It is commonly called as Madras Leaf-Flower and also called as Nila-Nelli in Tamil, Hajarmani in Hindi, Bhuiavali in Marathi, Nalla usirika in Telugu, Madaraas nelli in Kannada, kachora in Konkani and Bhumyaamalaki in Sanskrit.

The *Phyllanthus maderaspatensis* growing only 50 cm tall well-branched and hairless. Leaves are arranged in two ranks and they are inverted lance shaped, 1–4 cm long, up to 5 mm wide. They are expectorant, diaphoretic and useful in strangury and sweats. The seeds have a bad taste and are carminative, laxative, tonic to the liver, diuretic and useful in bronchitis, ear-ache, griping, ophthalmia and ascites. An infusion of the leaves is given for headache in South India.[3]

A number of the *Phyllanthus* species have been reported to have extensive history in medicine systems. Substantial amount of secondary metabolites present in the genus are used widely in traditional medicine for the treatment of flu, dropsy, diabetes, jaundice, gall and bladder calculus, liver disease.[4-6] Methanol extract of *Phyllanthus maderaspatensis* showed lowest antibacterial activity (3mm) against gram positive and gram negative bacteria.[7]

Aqueous extract of plant powder shown the remarkable hepatoprotective activity against acetaminophen-induced liver damage in Wistar rats at a dose of 500mg/kg [8] and also shown the same activity against cadmium- induced liver damage (200µg/kg).[9] The antimicrobial and hepatoprotective activity of *Phyllanthus maderaspatensis* may be due to the presence of phytochemicals. The *Phyllanthus* genus is a source of plant chemicals. Extracts of *Phyllanthus* has secondary compounds like alkaloid, flavonoid, lignin, phenol, tannin and terpene. Many of the "active" constituents are attributed to biologically active lignin, glycosides, flavonoids, alkaloids, ellagitannins and phenyl propanoids that are found in the leaf, stem and roots of the plant. Common lipids such as sterols and flavonols also occur in the plant.

The present study was carried out to evaluate the preliminary phytochemical and antimicrobial efficacy of the aqueous extract of Phyllanthus *maderaspatensis*, with the aimed of providing a lead compound(s) for the development of new, novel antimicrobial agent(s).

MATERIALS AND METHODS

Plant Material

Whole plant of *Phyllanthus maderaspatensis* (*Euphorbiaceae*) were collected from Urvara gardens, Kerala. The plant was authenticated by Botanical Survey of India, Coimbatore, India.

Plant Extraction

The collected plants were cleaned, dried under shade at room temperature and powdered. 10 gms of plant powder was treated with 100 ml of sterile distilled water (1:10) at room temperature with stirring. This procedure was repeated at least five times until the extraction solvent became colourless. The obtained extracts were filtered over Whatman No.1 filter paper and the filtrate was collected and then used as a test extract.

Phytochemical Screening

Preliminary phytochemical screening was performed to identify phytochemicals in the aqueous extract of *Phyllanthus maderaspatensis.* There are several sophisticated techniques e.g. Ultra Violet and Infrared Spectroscopy, Nuclear Magnetic Resonance and High-Performance Liquid Chromatography for identification of various groups of phytochemical compounds in plant extract; however, in the present work, the

phytochemicals were detected by colour tests and thin layer chromatography.

Colour Tests

The aqueous extract was screened for phytochemical constituents using standard procedure of analysis .[10-12]

Thin Layer Chromatography (TLC)

Silica gel coated TLC plates were purchased and was used for the study. A line was drawn on the TLC plate at a distance 2 cm from the base. Marks were made on the line for sample application. The sample was spotted on the line with the help of capillary tube and it was allowed to dry. The plate was placed in the developing jar with the mobile phase. When taken out of the jar, the solvent front was drawn. The plates were then kept in the iodine jar for a few seconds, shaken and taken out. They were examined under the UV/Vis lamp and the spots were circled with a pencil. The spots were labelled and their distances from the baseline were measured. The distance between the baseline and the solvent front was also measured. The R_f values were calculated.

Antimicrobial Activity

Microorganisms

The antimicrobial activity of the extracts was tested individually on gram-positive and gram-negative bacterial strains. All bacterial strains were obtained from the department of microbiology, Dr. N.G.P Arts & Science College, Coimbatore, India. The gram-positive bacterial strains used were Bacillus subtilis and Staphylococcus aureus and gram-negative bacterial strains used were Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhimurium. Bacterial strains were maintained on nutrient agar at 4^0C and sub-cultured every month in our laboratory.

Disc diffusion method [13]

Antibacterial activity was tested using a modification of the disc diffusion method. A loop of bacteria from the agar slant stock was cultured in nutrient broth overnight and spread with a sterile cotton swap into petri plates containing 10 ml of Mueller Hinton Agar. Sterile filter paper discs (9 mm in diameter) impregnated with the plant extract were placed on the cultured plates and incubated at 25 or 37^0C, depending on the bacteria. The solvent without extracts served as negative control. Standard antibiotic gentamicin (10 µg) was employed as positive control. After 24 h of incubation, the antibacterial activity was assessed by measuring the inhibition zone. The diameters of the zones of inhibition by the samples were then compared with the diameters of the zones of inhibition produced by the standard antibiotic discs. Each experiment was carried out in triplicate and the mean diameter of the inhibition zones was recorded.

Minimum inhibitory concentration (MIC) [14]

MIC is defined as the lowest concentration where no visible turbidity is observed in the test tube (Bacteriostatic concentration). MICs of the extracts were determined by the broth dilution method using serial dilution of the plant extracts. Briefly, the test bacteria were prepared in nutrient broth and incubated at 37 ^{0}C for 24 h. After that, the cultures concentrations were adjusted with sterilized saline to bring the optical density to 0.04 at 660 nm. Serial two-fold dilutions of the extracts were prepared in nutrient broth with concentrations ranging from 5.0000 to 0.0049 mg/ml. The 1 ml of each serially diluted extract was separately added to the tubes containing an equal volume of the inoculums. All the tubes were then incubated at 37 ^{0}C for 24 h. The tubes were then observed for visible growth with the help of a spectrophotometer. The MIC values were interpreted as the highest dilution of the sample, which showed clear fluid with no development of turbidity. Solvent blanks and positive controls were also included. All tests were performed in triplicate.

Minimum bacterial concentration (MBC) [15, 16]

The MBC is defined as the lowest concentration where no bacterial growth is observed (Bacteriocidal concentration). This was determined from the broth dilution resulting from the MIC tubes by sub culturing to antimicrobial free agar In this technique, the contents of the test tubes resulting from MIC was streaked using a sterile wire loop on agar plate free of bacteria and incubated at 37^0C for 18 hours. The lowest concentration of the extract which showed no bacterial growth was noted and recorded as the MBC.

RESULTS

The Phytochemical analysis of aqueous extract of *Phyllanthus maderaspatensis* showed the presence of saponins, carbohydrate, proteins, and tannins (Table1). TLC finger print profiles of this plant showed three bands at the R_f 0.05, 0.27 and 0.40 (Table 2). The results of the antibacterial activity and efficacies as compared to gentamicin are depicted in Table 3 shows that extracts exhibited appreciable antibacterial properties inhibiting the growth of all group of bacteria which were used for the study. At the concentration of 100 mg/ml aqueous extract of *Phyllanthus maderaspatensis* showed maximum activity (25mm) against *Staphylococcus aureus*. *Bacillus subtilis* formed less inhibitory zone (15 mm) when compare to other organisms.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) results are shown in Table 4 and Table 5. These tables reveal that the ranges of activity for both MIC and MBC are 25 to 75 mg/ml. It can also be seen that the MIC and MBC values of the extract on all the organisms is similar except *Staphylococcus aureus* (MIC value 25 mg/ml and MBC value 50 mg/ml).

DISCUSSION

Preliminary phytochemical tests of the whole plant of *Phyllanthus maderaspatensis* revealed the presence of secondary metabolites such as Tannins, Reducing Sugars, Proteins and Saponins, which is in conformity with the earlier reports on *P. Maderaspatensis*.[17] TLC analysis in aqueous extract revealed the presence of three spots. Each spot is probably due to a pure natural product or phytochemicals. Each also has a specific R_f value. The larger the R_f value, the lower the polarity of natural product/phytochemicals.

The results of the present study on the antimicrobial activity of *Phyllanthus maderaspatensis* against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Salmonella typhimurium* have shown that the aqueous extracts of the plant inhibited the growth of majority of the isolates. This is an indication that the extracts posses substances that can inhibit the growth of some micro-organisms. However, the observed inhibitory effects were more as the concentrations of the extracts increase 10 mg/ml, 25 mg/ml, 50 mg/ml, 75 mg/ml, and 100 mg/ml.

The antimicrobial properties exhibited by the extracts may be associated with the presence of these secondary metabolites through different mechanisms. Tannins form irreversible complexes with proline rich protein, resulting in the inhibition of cell protein synthesis. The MIC and MBC values are often near or equal in most bactericidal antimicrobials.[19] In our study we observed that all the microorganisms showing same MIC and MBC values except *Staphylococcus aureus*.

In the present study, results indicate that the aqueous extracts of *Phyllanthus maderaspatensis* possess antibacterial activity. These activities may be due to the presence of phytochemicals such as saponins, carbohydrate, proteins, and tannins which could serve as a lead to the isolation of chemotherapeutic agents. The isolation, identification, characterization and elucidation of bioactive compounds in this plant are ongoing and will be communicated later.

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Table 1: Phytochemical screening of aqueous extract of *Phyllanthus maderaspatensis*

S.No	Constituents	Inference
1	Alkaloids	-
2	Flavonoids	-
3	Saponins	+
4	Carbohydrate	+
5	Proteins	+
6	Phenols	-
7	Steroids	-
8	Glycosides	-
9	Resins	-
10	Tannins	+
11	Thiols	-

Key: + = Present, - = Absent

Table 2: TLC profile of aqueous extract of *Phyllanthus maderaspatensis*

Extract	No of spots visible by UV	R_f values
Aqueous extract of *Phyllanthus maderaspatensis*	3	0.05 0.27 0.40

Table 3: Antimicrobial activity of aqueous extract of *Phyllanthus maderaspatensis*

S.No	Microorganism	Zone of inhibition in mm*	
		Extract(100mg/ml)	Gentamicin
1	*Bacillus subtilis*	15±0.2	20±0.5
2	*Staphylococcus aureus*	25±0.9	21±0.2
3	*Escherichia coli*	20±0.5	24±0.4
4	*Pseudomonas aeruginosa*	20±0.8	20±0.1
5	*Klebsiella pneumoniae,*	17±0.2	24±03
6	*Salmonella typhimurium*	23±0.6	20±0.7

* Each value was expressed as the mean ± SD. (n=3)

Table 4: MIC values of aqueous extract of *Phyllanthus maderaspatensis*

S.No	Microorganisms	Extract concentration mg/ml					
		10	25	50	75	100	MIC
1	*Bacillus subtilis*	++	++	+	-	-	75
2	*Staphylococcus aureus*	+	-	-	-	-	25
3	*Escherichia coli*	+	-	-	-	-	25
4	*Pseudomonas aeruginosa*	++	++	+	-	-	75
5	*Klebsiella pneumoniae,*	++	+	-	-	-	50
6	*Salmonella typhimurium*	+	-	-	-	-	25

Key: - = No growth, ++ = Very turbid with growth, + = Slightly turbid

Table 5: MBC values of aqueous extract of *Phyllanthus maderaspatensis*

S.No	Microorganisms	Extract concentration mg/ml					
		10	25	50	75	100	MBC
1	*Bacillus subtilis*	++	++	+	-	-	75
2	*Staphylococcus aureus*	++	+	-	-	-	50
3	*Escherichia coli*	+	-	-	-	-	25
4	*Pseudomonas aeruginosa*	++	++	+	-	-	75
5	*Klebsiella pneumoniae,*	++	+	-	-	-	50
6	*Salmonella typhimurium*	+	-	-	-	-	25

Key: - = No growth, ++ = Very turbid with growth, + = Slightly turbid

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