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PHYTOCHEMICAL ANALYSIS, ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES OF *PHYLLANTHUS NIRURI*

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ABSTRACT
Phyllanthus niruri L. (Euphorbiaceae), Known as "quebra-pedra" (Portuguese for "stonebreaker") is an herb used for kidney disorders. Whole plants have been used in traditional medicine for treatment of jaundice, asthma, hepatitis and malaria. The present study was designed to determine the phytochemical analysis, antimicrobial activity and cytotoxicity of compounds isolated from the different solvent extracts of the aerial part of *Phyllanthus niruri*. The phytochemical analysis was done with 6 solvent extracts viz Hexane, chloroform, Acetone, Ethanol, Methanol and Aqueous). The phytochemical analysis revels presence various bioactive compounds like Alkaloids, Flavonoids, Tannins, Saponins, Glycosides, Terpenes, and Phenolic compounds in all the extracts at various concentrations. The Anthraquinones were absent in the plant extracts of all the solvents. The Antimicrobial activity was done against selected gram positive bacteria viz *Staphylococcus aureus*, *Bacillus subtilis*, Gram negative bacteria viz Klebsiella *pneumoniae and Proteus vulgaris*. Among all the solvents used in the study (hexane, Chloroform, Acetone, Ethanol, Methanol, Aqueous) the antibacterial efficiency of the methanolic extract of *Phyllanthus niruri* Linn. was high compare to other solvent extracts. The brine shrimp lethality test results reveal the LC50 value of methanolic extract was 0.8 µg/ml which proves its cytotoxic activity. Further the Anti-inflammatory studies were underway.

KEYWORDS: Antibacterial, Methanol, Inhibition zone, Cytotoxic.

INTRODUCTION
Plant-based antimicrobials represent a vast untapped source for medicines and further exploration of plant antimicrobials is needed as antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects of synthetic antimicrobials.[1] They may act as lead compounds for the pharmaceutical industry or as the base for the development of new antimicrobials.[2,3] Many of the plant materials used in traditional medicine are readily available in rural areas at relatively cheaper rates than modern medicines.[3] Medicinal plants represent a rich sources of secondary metabolites, many of which are antimicrobial agents.[4] Medicinal plants have been used since a long time as a source of medicine to combat various ailments including infectious diseases. According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs.[5] In developing countries, medicinal plants are used in traditional medicine. Such plants have been investigated for better understanding of their medicinal properties. Number of medicinal plants has been used for their antifungal and antibacterial properties.[6] and in the treatment of a wide range of infections.[7] *Phyllanthus niruri* Linn belongs to family Euphorbiaceae, commonly known as Stonebreaker (Eng.) due to its antilithic property. Various bioactivities such as antidiabetic.[8] anti-hepatotoxicity.[9] antilithic, anti-hypertensive, anti-HIV and anti-hepatitis B.[10,11] have been reported. Several studies have confirmed the antimicrobial efficacy of different *Phyllanthus* species; however, there is insufficient information regarding the antimicrobial and cytotoxic activities of methanolic extracts of *P. niruri*. Further studies on Anti-inflammatory activity were underway.

MATERIALS AND METHODS
Collection and identification of plant materials
The plant material *Phyllanthus niruri* were collected from the Dr. B.R. Ambedkar University Campus, Srikakulam district. The leaves were identified, confirmed and authenticated by comparing with an authentic specimen by a Botanist from Department of Botany, Andhra University. The plants were thoroughly washed with clean water to remove earthly matters and spread on clean surface in order to air dry. These were properly air dried for 1week at room temperature (37^0c). The plant material were dried under shade, segregated,

pulverised by a mechanical grinder and passed through a 40 mesh sieve.[12]

Extraction of the Plant Material

The whole plant materials were air dried until all the water molecules evaporated and plants become well dried for grinding. After draying, the plant material were grinded well using mechanical blender into fine powder and transferred in to air tight containers with proper labelling for future use. The ethanol, methanol and aqueous extracts were prepared by 100gms of powdered plant material soaked in 500ml of different solvents in room temperature at 72h. The extracts were filtered through muslin cloth and through what men filter paper (Grade 1). Extracts are concentrated by using water bath contains rotary evaporator. Total yield of plant extract ranges from 5 -6% respectively.

Phytochemical Analysis

Qualitative analysis of the crude extracts were carried out as described by Brain and Turner, Sofowora , Trease and Evan, Ushie and Adamu and Ushie *et al.*,[13,14,15,16] to identify the presence of the classes of Secondary Metabolites (Alkaloids, Anthraquinones, Flavonoids, Tannins, Saponins, Glycosides, Terpenes, Phenolic compounds).

Test for Alkaloids

0.5 g of the extract was stirred with 2 M aqueous hydrochloric acid (5.0 ml) on a steam bath. 1.0 ml of the filtrate were separately treated with a few drops of Mayer s reagent, Drangendoff s' reagent, Wagner s reagent. The resulting solution was observed for colour changes.

Test for Tannins

0.5 g of each of the plant extracts was boiled with distilled water (100 ml) for 5 minutes. To 2.0 ml of the cooled solution (filtrate) a few drops of ferric chloride was added. The colour change was recorded.

Test for Glycosides

A small portion of each of the plant extracts was placed in two separate test tubes of 0.1 M H_2SO_4 was added to one and distilled water (5.0 ml) added to the other. The test tubes were heated for 45 minutes in a water bath. The cooled solutions were made alkaline with a solution of 2 M NaOH. Fehling solutions (5.0 ml) A and B (ratio1:1) was added to the two test tubes and were allowed to stand for 3 minutes. The solution of the extracts in distilled water serves as control. The changes in reaction were observed and recorded.

Test for Saponins

The froth test and emulsion test as described by Harborne[17] were used to determine the presence of saponins. A small portion of each of the plant extracts was added to distilled water (20 ml) in a 100 ml beaker, boiled and filtered and the filtrate used for the test.

(a) Froth test: 5 ml of the filtrate was diluted with water (20 ml) and shaken vigorously and allowed to stand for 30 minutes. The result was recorded.
(b) Emulsion test: 2 drops of olive was added to the frothing solution and shaken vigorously. The result was recorded.

In order to remove 'false-positive", the blood haemolysis test was performed on the extract that frothed water.

Test for Anthraquinones

0.5 g of each of the plant extracts was shaken with benzene (2.0 ml) and filter where necessary. 10% ammonia solution (4.0 ml) was added to the filtrate. The resultant mixture was shaken and the reaction observed and recorded.

Test for Flavonoids

(a) Lead Acetate Test: 0.5g of the extract dissolved in 5 ml of distilled water. 10% of lead acetate solution (1.0 ml) was added. The colour formation was recorded.
(b) Iron (111) chloride. To a solution of 0.5 g of the extract in water, two drops of iron (111) chloride was added. A colour change noted and recorded.

Test for Terpenoids (Salkowski test)

A solution of each of the extract was made by dissolving 0.5 g of the extract in 2.0 ml of chloroform and concentrated H2SO4. The presence of terpenes in the sample was detected as the colour changes**.**

***Test for Phenolic compounds* (Ferric chloride test)**

A fraction of the extracts was treated with aqueous 5% ferric chloride and observed for formation of deep blue or black colour.

Screening of Plants extracts for Antimicrobial Activity

Microbial cultures

In this study, Microorganisms were selected to cover Gram-positive bacteria and Gram-negative bacteria. All the microbial cultures were obtained from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh. *Bacillus subtilis* (MTCC-441)**,** *Proteus vulgaris* (MTCC-1771), *Stphylococcus aureus* MTCC-766 and *Klebsiella pneumoniae* MTCC-109 were used for antimicrobial study. The cells from lyophilized vials were transferred into the liquid nutrient broth medium, and then transferred into nutrient agar slants preserved at 37°C in the refrigerator. The Microorganisms were allowed to grown over night at 37^0C on nutrient agar at pH 7 prior to antimicrobial screening.

Standardization of Inoculums

The seven test organisms were sub-cultured with nutrient broth using a wire loop (done aseptically) and incubated for 24 h at 37^oC for bacteria. The growth of the micro-organisms in the broth by the turbidity produced was adjusted to match 0.5 McFarland standards (108cfu/ml), which was further adjusted to 103cfu/ml.

Inoculation of the Plates and Application of the Extracts

The screening of Anti-microbial efficacy of the various extract of Phyllanthus niruri was performed on various Microorganisms by using Agar well diffusion method. The agar plates were prepared by pouring 20 mL of sterile molten Nutrient Agar (Himedia Lab Pvt. Ltd, Mumbai, India). The bacterial cultures were prepared by adding the seed culture in the autoclaved agar medium followed by pouring into Petri plates. One microbe was inoculated to one plate. The solid agar medium was gently punctured with the aid of 8mm sterile cork borer to make a proper well. Seven wells for hexane, chloroform, ethyl acetate, acetone, methanol, aqueous extracts and control (Tetracycline) were made. The plant extracts is diluted using dilution method and in each of the appropriately labelled well (hole) diluted plant extract were introduced. Tetracycline was also introduced in the well as control. The diffusion of extract was allowed for 1hr at room temperature on a sterile bench. Then the Petri plates were incubated for 48 hrs. at 37^0C. After 48 hrs. the plates were observed for the presence of inhibition of bacterial growth and that was indicated by clear zone of inhibition of bacterial growth around the wells. The diameter of zones of inhibition was measured by means of linear instrument in millimetre (vernier calliper) and recorded. The size of Inhibitory zone was measured in mille meters (mm). Minimum Inhibitory Concentration (MIC) was determined.

Brine shrimp lethality test

The cytotoxicity activity of the crude extract was assessed on brine shrimp nauplii (*Artemia salina*) according to brine shrimp lethality bioassay.[18] Artificial sea water was prepared by dissolving 12 g sodium chloride in 1 L of distilled water and adjusting the pH to 8.5 using 40% sodium hydroxide. Some 2 g of brine shrimp eggs were hatched in 1L of sterile sea water in a flask. The cysts were kept under bright light, and were continuously agitated and aerated using an aquarium pump. The nauplii hatched within 48 h at room temperature. The methanol crude extract was dissolved in 1% aqueous dimethyl sulfoxide (DMSO) in artificial sea water to obtain concentrations of extract varying from 100 mg/ml to 0.25 mg/ml. Some 50 µl of each solution was transferred, to clean sterile vials containing 5 ml of aerated seawater. Ten shrimp nauplii were transferred to each vial. The experiments were performed in triplicate for each extract concentration. Potassium dichromate (5mg/ml) and 1% DMSO in seawater were used as positive and negative controls, respectively. After 24h, the vials were examined against a lighted background using a hand held magnifying glass and the number of nauplii that survived in each vial were counted.

RESULTS AND DISCUSSION

Investigations on the phytochemical screening of *P. niruri* ethanolic extract revealed the presence of alkaloids, terpenoids, tannins, saponins, quinones, glycosides, phenols, reducing sugars, flavonoids, resins, steroids and anthraquinones. However in the methanolic extract all the phytochemicals are seen, except anthraquinones. Where as in aqueous extract resins and anthraquinones are absent. These phytochemicals are biologically active. These metabolites can exert antimicrobial activity through different mechanisms (Table 1). The antimicrobial activity of ethanolic, methanolic and aqueous extracts of *Phyllanthus niruri* was investigated by using agar diffusion method against selected gram positive and negative bacteria.

The antibacterial activity of the plant extracts from *phyllanthus niruri* was studied against bacterial MTCC strains *Staphylococcus aureus* (MTCC-766), *Klebsiella pneumoniae* (MTCC-109), *Bacillus subtilis* (MTCC-441) and *proteus vulgaris.*[19,20,21] using agar well diffusion method (Figure-2) The result revealed that inhibitory effects of test samples was dose dependent as the concentration increased the zone of inhibition was also increased. This is also presence of antibacterial activity of *Phyllanthus niruri* against gram positive and gram negative bacteria by dose dependent manner. As shown in Table-2 The methanol extracts of the whole plant showed maximum activity against *Bacillus subtilis* followed by *Klebsiella pneumoniae, proteus valgaris* and *Staphylococcus aureus.* The acetone extracts of this plant (leaves, stem and fruits) showed maximum activity against *Staphylococcus aureus.* followed by *Klebsiella pneumoniae, proteus valgaris and Bacillus subtilis* and Ethanolic extracts of this plant (leaves, stem and fruits) showed maximum activity against *Staphylococcus aureus* followed by *Klebsiella pneumoniae, proteus valgaris and Bacillus subtilis.*[22,23] The chloroform extract of the whole plant showed maximum activity against *Staphylococcus aureus* followed by *Klebsiella pneumoniae, Bacillus subtilis, and proteus valgaris.* The antibacterial activity of methanol extract showed more effective followed by ethanol, acetone, chloroform, Hexane, Aqueous extracts against all the bacterial strains. All the experiments were conducted in triplicate and results were recorded and tabulated as mean values.

The brine shrimp mortality was 100% in the Potassium dichromate standard and in all extract concentrations above 8 µg/ml. The mortality of brine shrimps increased with increasing concentration of extract. The regression equation on Figure 1 was used to calculate an LC50 value of 0.8 µg/ml. Standard brine shrimp lethality bioassay stipulates that LC50 values less than 1000 µg /ml are considered bioactive in toxicity evaluation of plant extracts.[20] Based on this benchmark, the *P. niruri* extract is strongly cytotoxic since the LC50 value was less than 1000 µg/ml. Cytotoxicity of the extract is attributed to presence of alkaloids, tannins, flavonoids and terpenoids.[24.25] The LC50 value of 0.8 µg/ml for *P.niruri* is comparable to that of other Phyllanthus species which has been attributed to presence of Terepenoids. Alkaloids etc.[26,27,28]

The present study has demonstrated that the whole plant methanolic extract of *Phyllanthus niruri* exhibits good Antimicrobial activity and cytotoxicity activities. Caution should however, be exercised in the use of this species for medicinal purposes because of its high toxicity as evidenced by the LC50 values of 0.8µg/ml. The results also suggest that the plant contains bioactive constituents that are responsible for the observed activities. Further there is a need to study anti-inflammatory studies which are underway.

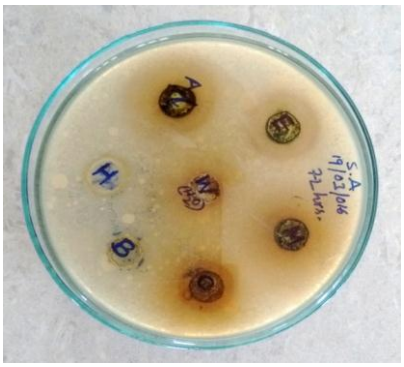
Table1: Phytochemical screening of *Phyllanthus niruri* in various solvent extracts

Phytochemicals	Hexane Extract	Chloroform extract	Ethanolic extract	Acetone extract	Methanolic extract	Aqueous extract
Alkaloids	++	++	+++	++	+++	++
Tannins	++	++	+++	++	+++	++
Glycosides	++	++	+++	++	+++	++
Saponins	++	++	+++	++	+++	++
Anthraquinones	-	-	-	++	-	-
Flavonoids	++	++	+++	++	+++	++
Terpenoids	++	++	+++	++	+++	++
Phenolic compounds	++	++	+++	++	+++	++

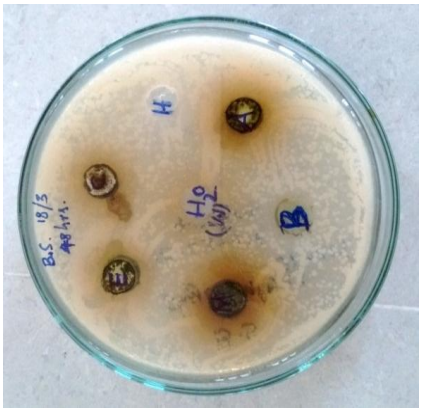
Table 2: Antibacterial activity of *Phyllanthus niruri* in different solvent extracts

Microorganism	Inhibition zone diameter(mm)					
	Hexane Extract	Chloroform extract	Ethanolic extract	Acetone extract	Methanolic extract	Aqueous extract
B. subtilis (MTCC-441)	12	14	18	14	18	12
S.aureus (MTCC-766)	14	15	22	14	24	9
P.vulgaris (MTCC-1771)	12	10	18	12	20	14
K. pneumoniae (MTCC-109)	12	9	18	12	22	15

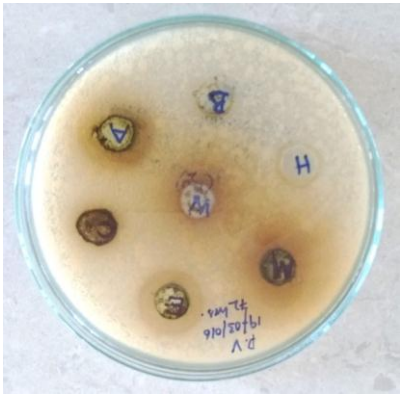
Figure 1: Phyllanthus niruri



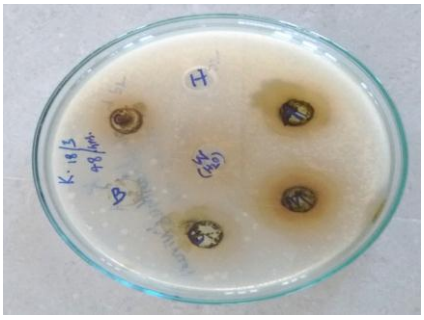
Staphylococcus aureus



Bacillus subtilis



Proteus valgaris



Klebsiella pneumonia

Figure 2: Antimicrobial activity of *Phyllanthus niruri* shown by agar diffusion method

CONCLUSION

The present findings can be of commercial interest to both pharmaceutical companies and research institutes in the production of new antimicrobial drugs. More importantly, there have been no side effects or toxicity reports from many years on this plant. There is still a lot of scope for further research, especially towards the mechanism of biological activity of phytochemicals from this plant.

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