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SCREENING AND EVALUATION FOR ANTIBACTERIAL AND ANTIOXIDANT POTENTIALS IN STEM EXTRACT OF *DERRIS TRIFOLIATA* L

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Abstract: Objectives: Stem infusions of *Derris trifoliata* L (DT) finds a prominent place in folk medicine. In this study, we qualitatively screened the ethyl acetate, acetone, methanol and ethanol extracts of DT stem for secondary metabolites like alkaloids, flavonoids, saponins, and tannins. Biological activities such as antioxidant and antibacterial potentials were also quantified in these extracts. Methods: Phytochemicals in the respective solvent extracts of DT were determined by standard procedures. Total antioxidant activity was quantified Spectrophotometrically by FRAP assay. Antibacterial strength was assessed against selected 6 bacterial species *Escherichia coli* (MTCC 7410), *Enterobacter aerogenes* (MTCC 7324), *Enterobacter cloacae* (MTCC 7408), *Bacillus subtilis* (MTCC 736), *Enterococcus faecalis* (MTCC9845), and *Streptococcus pyogenes* (MTCC 1928) by agar well diffusion method and compared with gentamicin and tetracyclin. The Minimum Inhibitory Concentration [MIC] was determined by serial dilution method. Results: Phytochemical screening showed that the crude extracts were found to be positive to alkaloids, flavonoids, saponins and tannins. Antioxidant activity of the extracts was expressed as FRAP units and it varies amid the solvents used. Methanol extracts showed highest level of antioxidant activity (632), while ethyl acetate extract was identified to contain lower antioxidant activity (196). Extracts of DT exhibited different degrees of anti bacterial activity and varied from solvent to solvent. Among the extracts, methanol extract exhibited maximum antibacterial activity against the tested gram positive species than that of gram negative cultures. The MIC values were found to be between 1.25 to 5mg/100µl. Conclusion: This study provides the necessary data for isolation and characterization of bioactive principles that possess the antioxidant and antibacterial activity from different extracts of DT stem.

Keywords: *Derris trifoliata*; Secondary metabolites, Antibacterial activity, Antioxidant activity, Minimum Inhibitory Concentration

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INTRODUCTION

Plants have been a rich source of phytomedicines because they produce an array of bioactive molecules, most of which probably evolved as chemical defense against predation and/or infection. The use of plant extracts and phytochemicals, with known antibacterial properties, are important in therapeutic applications. According to the WHO plants would be the best source of obtaining a variety of drugs. About 80% populations of the developed countries use traditional medicines that are derived from plants. Antibiotic resistance has become a global concern[4]. There has been an increasing incidence of multiple resistances in human pathogenic microorganisms in recent years, largely due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. The search for plants with antimicrobial activity has gained increasing importance in recent years due to growing worldwide concern about the alarming increase in the rate of infection by antibiotic resistance microorganisms. Adverse effects associated with chemotherapy draw the attention of the phyto-chemists globally towards the search of new drugs to combat the challenges posed by resistant strains of microorganisms. A large number of plant infusion indeed were used to control different diseases and known to possess antimicrobial activity[1,2]. The investigation of certain indigenous plants for antimicrobial properties provided valuable information. It is believed that plant based drugs cause less or no side effect when compared with synthetic antibiotics[3]. Mangroves are perennial plants that grow in coastal wetlands of tropical regions. Mangroves are unique in that they produce an array of novel natural products. Mangroves and their products are known to be associated with biologically active antiviral, antibacterial and antifungal compounds[5]. Mangrove plants and plant products have been used for centuries as natural remedies in the treatment of several health disorders[6]. Systematic study of mangrove species has revealed that crude extracts of different plant parts in different solvents and compounds isolated from them exerted potential antibacterial, antifungal, antiviral and antioxidant activities[7]. *Derris trifoliata* L belongs to the family *fabaceae*. It is perennial climber grows up to 8m. Aerial parts of the plant traditionally used against diarrhea and dysentry[8]. In the present study, we examined different extracts prepared from the stem of *Derris trifoliata* for their secondary metabolites as well as antioxidant and antibacterial activity.

MATERIALS AND METHODS

Collection of Plant material

This plant grown in the areas where soil is sandy and less saline .It is a large, woody, climbing shrub, branches are wiry. The plant part was collected from Corangi Reserved Forest, Kakinada, East Godavari, Andhra Pradesh, India. Geographic location is between 16^o 39' N longitude - 17^o N longitude and 82^o 14' E latitude - 82^o 23'E latitude. All the stems were surface sterilized with

1% mercuric chloride solution and thoroughly washed with filter sterilized distilled water[9]. The washed stems were then chopped to small pieces and shade dried until they were suitable for extraction in the selected solvents.

Extraction

Plant extracts in ethyl acetate, acetone, ethanol and methanol were prepared according to the standard method[10]. The chopped stem material (100g) was initially soaked in 500ml of the respective solvent at room temperature for 24h. Subsequently, the soaked material was refluxed for 6h below the boiling point of the respective solvent. Infusions were filtered through Whatman No.1 filter paper and the residual material was re-extracted with fresh solvent. After 24h the process was repeated. Pooled extracts were individually concentrated by removing the solvent under reduced pressure and temperatures using vacuum rotator evaporator. These extracts were further concentrated by solvent evaporation using thin film method. Dried fruit extract of 100mg each was dissolved in 10ml of 1:10 diluted DMSO in sterile distilled water so as to obtain the final concentration of 10mg/ml [11]. All the extracts thus prepared were stored in a refrigerator at 4^{o}C.

IDENTIFICATION OF SECONDARY METABOLITES.

The crude extracts in ethyl acetate, acetone, ethanol and methanol of DT were qualitative screened for secondary metabolites viz., alkaloids, flavonoids, saponins, and tannins by using standard protocols[12,13].

Test for Alkaloids

5 ml of the crude extracts was stirred with 10 ml of 1% aqueous HCl in water bath and then filtered. To 2ml filtrate 4-6 drops of Dragendroff's reagent was added. Formation of orange – red precipitate was considered as positive to alkaloids.

To 2 ml filtrate few drops of Mayer's reagent was added and appearance of buff-coloured precipitate was taken as existence of alkaloids.

Test for Flavonoids

About 5ml of the test solution was boiled with 10 ml of distilled water and then filtered. Later, 2 ml of lead acetate solution was added to 2 ml of the filtrate. Appearance of buff coloured precipitate considered as positive to flavonoids.

To 2 ml of the filterate, 5 ml of dilute ammonia solution was added followed by 4–6 drops of concentrated sulphuric acid. Appearance of yellow color indicates the presence of flavonoids.

Test for Saponins

About 5 ml of crude extract was shaken with 5 ml of water in a test tube and it was warmed in a water bath. The persistent froth indicates the presence of saponins.

Test for tannins

About 5ml of each extract was stirred with about 10ml of distilled water and then filtered. Few drops of 1% ferric chloride solution was added to 2ml of the filtrate. Occurrence of a blue-black, green or blue-green precipitate indicates the presence of tannins.

About 5 ml of each extract was added with 1 ml of 1% HCl solution. Formation of red precipitate indicates the presence of tannins.

DETERMINATION OF ANTIOXIDANT ACTIVITY BY THE FRAP METHOD

Total antioxidant capacity of all the extracts of DT were measured by using FRAP method[14]. The FRAP method (Ferric Reducing Antioxidant Power) is based on the reduction of complexes of 2,4,6-tripyridyl-*s*-triazine (TPTZ) with ferric chloride.

Reagent preparation: FRAP reagent was prepared freshly by mixing 25ml of acetate buffer (pH 3.6) with 2.5 ml of 10mM 2,4,6 tripyridyl triazine (TPTZ) and 2.5 ml of 20mM Ferric chloride solution. The reagent was kept at 37^{o}C before its use.

Procedure: Exactly 300µL of the stem extract (100mg/1000µL) was dispensed into 2700µL of the freshly prepared FRAP reagent and incubated at 37^{o}C for 30min. The absorbance was recorded at 593nm against the blank. Standard curve was prepared with 100µM $FeSo_4$ solution. All the tests were performed in triplicates and the mean values were expressed as µM Fe^{2+} equivalents per 100 gram of the sample.

DETERMINATION OF ANTIBACTERIAL ACTIVITY

Antibacterial activity of all the extracts prepared in different solvents from dried stem sample of DT was determined using standard agar well diffusion method [15-17]. Pure cultures of *Escherichia coli* (MTCC 7410), *Enterobacter aerogenes* (MTCC 7324), *Enterobacter cloacae* (MTCC 7408), *Bacillus subtilis* (MTCC 736), *Enterococcus faecalis* (MTCC 9845) and *Streptococcus pyogenes* (MTCC 1928) were selected to measure the antibacterial potential of all the extracts of DT. Each experiment was performed in triplicates and the average zone of inhibition was calculated. The zones were compared with that of the broad spectrum antibiotics Gentamicin and Tetracyclin at a concentration of 10µg/disc[18].

DETERMINATION OF MIC

Minimum Inhibitory Concentration [MIC] was determined by broth dilution assay method[19]. Plant extracts were serially diluted in Mueller Hinton broth to get the concentrations of 1.25, 2.5, 5.0 and 10mg/100µl. Each experiment was repeated thrice and the mean values were tabulated.

RESULTS

Phytochemical screening of all the extracts of DT for secondary metabolites shows that all the extracts were found to be positive to all the tested secondary metabolites except ethyl acetate extract for saponins as shown in Table 1. This secondary metabolite diversity indicates that *Derris trifoliate* survives in tough environmental conditions and survives by protecting itself from assorted pests and pathogens.

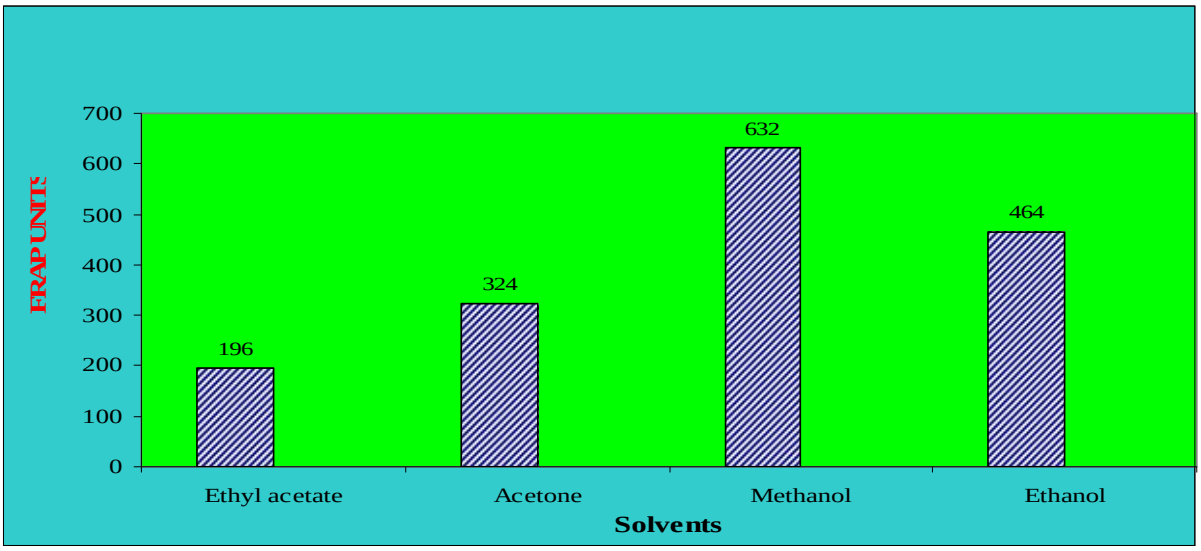
Table 1. Phytochemical analysis of extracts of *Derris trifoliata*

	Alkaloid	Flavonoid	Saponin	Tannin
Ethyl acetate	+	+	-	+
Acetone	+	+	+	+
Methanol	+	+	+	+
Ethanol	**+**	**+**	**+**	**+**

ANTIOXIDANT ACTIVITY

Several techniques have been used to determine the antioxidant activity. Free radicals are known to play a definite role in a wide variety of pathological conditions. The non enzymatic antioxidant activity of plants is authorized to their quality and quantity of secondary metabolites. The antioxidant activity of all the fractions was estimated spectrophotmetrically by FRAP method and the data was diagrammatically presented in Fig-1. The outcome of antioxidant activity by FRAP method reveals that methanol soluble exhibited maximum activity followed by ethanol, acetone and ethyl acetate. These results correlate with the amount of secondary metabolites present the stem extracts of *Derris trifoliate* in ethyl acetate, acetone, methanol and ethanol. Antioxidants fight against free radicals and protect us from various diseases.

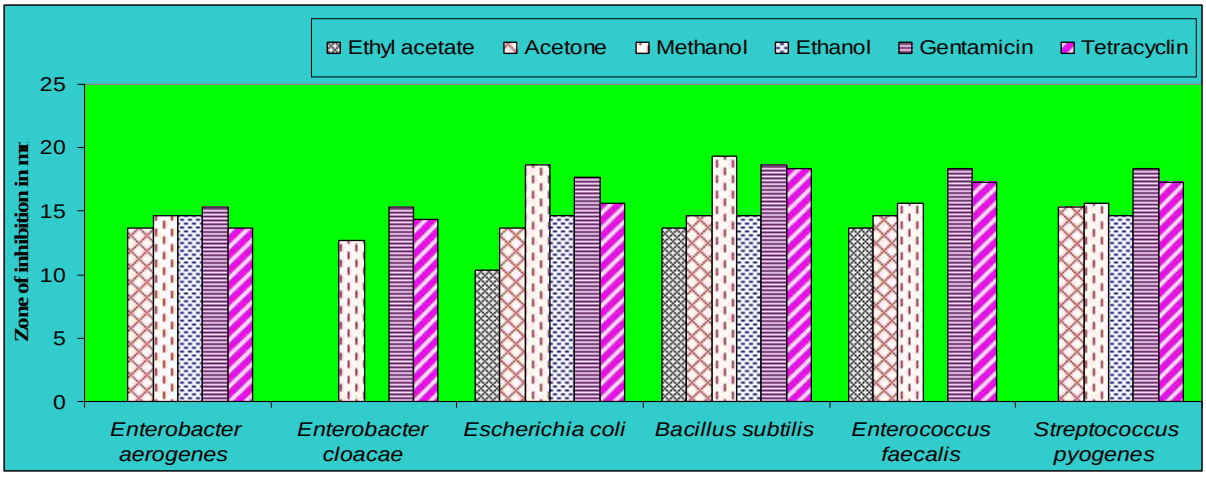
Fig-1 Antioxidant activity of *Derris trifoliata* by FRAP method.



ANTIBACTERIAL ACTIVITY

In vitro antibacterial activity of all the stem extracts of DT in organic solvents in the increasing polarity was quantified by agar well diffusion method and the data was systematically presented in Figure-2. All the extracts were found to possess various degrees of antibacterial activity against both Gram positive and Gram negative bacteria. Among the tested extracts bioactive molecules extracted into methanol exhibited highest antibacterial activity (12.66-19.33mm) against all the tested bacterial species irrespective of their Gram nature. Active constituents of ethyl acetate fraction specifically inhibited the growth of *E. coli, B. subtilis, En. faecalis.* Acetone solubles of DT exerted more or less similar activity against all the tested cultures except no activity against *En. cloacae*. The last solvent used in the order of polarity is ethanol and the principle constituents were found to be active against 2 Gram positive and 2 Gram negative cultures. Crude extract of DT in methanol exhibited significantly higher zone of inhibition against *E. coli* and *B. subtilis,* as compared to that of the standard antibiotics gentamicin and tetracycline. This clearly indicates that methanol as an appropriate solvent to isolate active broad spectrum antibacterial molecules of DT.

Fig-2 Antibacterial activity of *Derris trifoliata* against the test organisms.



Further, these extracts were analyzed to determine the minimum inhibitory concentration against both Gram negative and Gram positive bacterial species. The MIC values ranged from 1.25-5mg/100µl and varied from extract to extract as presented in table-2. The MIC of acetone and methanol extracts towards the tested cultures is 5mg/100µl. Where as, the MIC of methanol extract is 1.25mg/100µl to *Escherichia coli* and *Bacillus subtilis*, while the MIC value to the remaining test culture is 2.5mg/100µl.

Table-2: MIC of *Derris trifoliata* stem extracts (mg/100µl)

Microorganisms	Ethyl Acetate	Acetone	Methanol	Ethanol
Escherichia Coli	-	5	2.5	5
Enterobacter aerogenes	-	0	2.5	-
Enterobacter cloacae	5	5	1.25	5
Bacillus subtils	2.5	5	1.25	5
Enterococcus faecalis	2.5	5	2.5	-
Streptococcus pyogenes	-	5	2.5	5

DISCUSSION

Plants are important source for the isolation and development of novel, potential drugs and new lead molecules. Plant based antimicrobials and antioxidants represent a diverse group of molecules employed in the control of multi drug resistant bacterial diseases and to rejuvenate the antioxidant capacity [20]. Secondary metabolites such as alkaloids, flavonoids, steroids, saponins tannins and phenolic compounds are synthesized and deposited in all parts of the plant are responsible for many bioactive properties. Over 5000 naturally occurring phenols and

flavonoids have been identified from various plants and some are known to associate with diverse biological activities .

Mangroves are assorted group of plants rich with many secondary metabolites like alkaloids, flavonoids, phenolics, steroids and terpenoids [21,22]. They are well studied for their pharmacological and ecological importance. In folk medicine mangrove plant parts and extracts are being used in the treatment of various diseases over the centuries .These are known to possess antioxidant, antibacterial, antitumor and antiviral properties. Phytochemical constituent found in the extract of DT was previously reported [23] . Our study reported that methanol extract of the stem obsessed most of the secondary metabolites. This data also reveals that the secondary metabolites infused into different organic solvents are found to be polar.

Khan *et al* screened various parts of *Derris elliptica, Derris indica* and *Derris trifoliata* in different organic solvents and demonstrated different degrees of antibacterial activity [24]. The present work brings additional information of the antibacterial activities of DT against 3 Gram positive and 3 Gram negative bacterial species.

In search of potent broad spectrum antibacterial compounds, we have screened the inhibitory effect of DT stem extracts in ethyl acetate, acetone, methanol and ethanol against selected microorganisms viz., En *aerogenes, En cloacae, E coli, B subtilis, En faecalis, and Strep pyogenes*. All the tested extracts of DT were found to possess different amounts of antibacterial activities (10.33-19.33mm). Methanol extracts exerted highest antibacterial activity without Gram restriction. Hence it is concluded that the active constituents of methanol might be considered as the potential source of broad spectrum antibacterial compounds. The zone of inhibition exhibited by the methanol extracts was slightly higher towards gram positive cultures over gram negative cultures. The difference in rate of inhibition appears to be directly related to the quality and quantity of the compounds present in the extract. These results are in agreement with the findings of Khan *et al*. Extracts of DT in ethyl acetate, acetone and ethanol inhibited effectively all the tested Gram positive bacterial strains when compared with that of the Gram negative cultures. This may be due to the permeability factor of cell membrane of the microorganism[25], or this could be due to variation in the cell wall composition of Gram negative and Gram positive bacteria. The Gram negative bacteria restrict the influx of many antibiotics. Multi drug efflux pumps at the trans-membrane are also responsible for a higher intrinsic resistance in Gram negative bacteria [26]. On correlating our results of secondary metabolites and antibacterial activities it is inferred that alkaloids, flavonoids, saponins and tannins possess substantial antibacterial activity. Ethyl acetate extracts did not inhibit *En. aerogenes, En. cloacae*. These negative results did not mean absence of bioactive principles in the ethyl acetate extract. The bioactive principle may be insufficient to cross the membrane, or

the microorganism may associate one or more resistant mechanisms to neutralize the activity of the bioactive molecules present in the extract. The inhibitory effect of gentamicin is superior to that of the tetracycline and our results are in agreement with the study of Odubanku [27] and Suganya [28]

In our study, the MIC value for all the positive extracts against the tested bacteria were between 1.25mg/100µl to 5mg/100µl. Gram negative test cultures showed higher MIC values than Gram positive text cultures. However, MIC values of stem extracts in acetone and ethanol are higher for all the test culture than the methanol extract. This difference may be explained by susceptibility testing condition, physic-chemical characters of the bioactive principle present in the extract and even strain to strain difference. In comparison to some of the earlier reports on MIC values of pure compounds, our MIC may be higher, but this can be substantiated by the argument that this value is for the crude extract[29,30]. Fai-Chu Wong [31] studied the MIC on *S. aureus, M.luteus, E.coli* and *P.aeruginosa* with ampicillin and selected medicinal plant extracts and reported the MIC value of Amphicillin is between 0.02 - 1mg/1000µl and that of the plant extracts are in the range of 6.3 – 50 mg/1000µl. Our results are far superior compare to that of the plant extracts but inferior to that of the standard antibiotic.

The antioxidant activity is high for methanol extract and it correlated with the phytochemical studies. As the methanol extracts contains all the screened secondary metabolites. The present experimental data is in accordance with that of Govindasamy *et.al* [32] who studied the chemical constituents of different mangroves in India. The role of alkaloids, flavonoids and tannin in scavenging of free radicals has been well studied [33,34]. These are also known to be associated with other biological activities such as antibacterial, antifungal, antidiuretic activities [35,36]. Hence, all the plant parts of *Derris trifoliate* are strongly recommended for the isolation of potent bioactive principles exhibiting antioxidant and antibacterial activity.

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