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Research Paper

Extraction of Flavonoids and Tannins in Roots of Derris Trifoliata L and Evaluation for Antibacterial and Antioxidant Potentials

Microbiology

KEYWORDS : Derris trifoliata, Flavonoid, Tannin, Antibacterial activity, Antioxidant activity, Minimum Inhibitory concentration

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ABSTRACT

The plant extracts of Derris trifoliata L finds a prominent place in folk medicine. In this study, we extracted and quantified flavonoids and tannin from the roots of Derris trifoliata in ethyl acetate and methanol, them evaluated their antioxidant activity by FRAP assay and antibacterial activity by agar well diffusion method. The bacterial species are Enterobacter aerogenes , Enterobacter cloacae, Staphylococcus aureus and Streptococcus pyogenes were compared with standard antibiotic gentamicin. The Minimum Inhibitory Concentration [MIC] was determined by serial dilution method. The methanol extracts showed highest levels of antioxidant activity as well as the inhibitory effect on the test cultures used. The MIC value was found to be between 2.5 to 5mg/100µl. This study provides the necessary data for extraction and characterization of bioactive principles that possess the antioxidant and antibacterial action from ethyl acetate and methanol root extracts of Derris trifoliata

INTRODUCTION

Plants have been a rich source of medicines because they produce wide array of bioactive molecules, most of which probably evolved as chemical defense against predation or infection. The search for plants with antimicrobial activity has gained increasing importance in recent years due to a growing worldwide concern about the alarming increase in the rate of infection by antibiotic resistance microorganisms [1]. 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 [2]. This has forced the scientific community to look for novel antimicrobial substances from plants for the treatment of infectious diseases. Many studies point out that plants hold many bio-active compounds such as alkaloids, flavonoids, glycosides, saponins, terpenoids, etc with antimicrobial, antioxidant, antidiuretic activity. The term mangrove is also used to designate halophytic (salt loving) and salt resistant marine tidal forests comprising of trees, shrubs, palms, epiphytes, ground ferns and grasses. Mangroves occur in 121 countries covering 18 million hector worldwide. Globally, mangroves are distributed across the tropical and subtropical forests and are predominantly found in tropical region. Asia and Australia have the greatest diversity and distribution of mangrove species in the world. The major mangrove wetlands of India are located along the East Coast. Systematic study of mangrove species has revealed that crude extracts of different plant parts of mangroves in different solvents make use of potential antibacterial, antifungal, antiviral and antioxidant activities[3]. Derris trifoliata L belongs to the family fabaceae. It is perennial climber grows up to 8 meters . Aerial parts of the plant traditionally used against diarrhea and dysentery[4]. In the present study, we examined extracts prepared in ethyl acetate and methanol from the roots of Derris trifoliata L for the flavonoid and tannin content as well as their antioxidant and antibacterial activity. .

MATERIALS AND METHODS

Collection of Plant material

This plant grows in the areas where soil is sandy and less saline. It is a large, woody, climbing shrub, branches are wiry. The plant part were collected from Corangi Reserved Forest, Kakinada, East Godavari, Andhra Pradesh, India. Geographic location - between 16° 39' N longitude - 17° N longitude and 82° 14' E latitude - 82° 23'E latitude. All the roor were surface sterilized with 1% mercuric chloride solution and thoroughly washed with filter sterilized distilled water[5]. The washed root, were then chopped to small pieces and shade dried until they were suitable for extraction in the selected solvents.

ISOLATION

Plant extracts in ethyl acetate and methanol were prepared according to the standard protocols [6]. The chopped root 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 Whattman 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 temperatures using vacuum rotator evaporator. These extracts were further concentrated by solvent evaporation using thin film method.

EXTRACTION OF FLAVONOID

Flavonoids were isolated by Bohan and Kocipai-abyazan method [7]. One gram of the individual ethyl acetate and methanol plant extract sample was re-extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered by whattman filter paper. The filtrate was concentrated with vacuum rotor evaporator and transferred in a crucible and further evaporated to dryness on a water bath and weighed to a constant weight.

EXTRACTION OF TANNIN

1g of individual extract was shaken with 100 ml of ethanol on mechanical shaker for 30 minutes. 10% lead acetate solution is treated to precipitate tannins as lead tannate. The suspension was separated from the solvent by centrifugation. The precipitate was suspended in ethanol and hydrogen sulphide gas is passed to remove lead as lead sulphide, the resultant light yellow coloured filtrate was evaporated to dryness on water bath to constant weight [8]. The extracts 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. All the extracts thus prepared were stored in a refrigerator at 4°C.

DETERMINATION OF FLAVONOID

Total flavonoid content in the plant extracts were measure following the aluminium chloride method of Mariniva *et al* [9]. 1ml of plant extract (10mg/ml) was diluted with 4ml of distilled water in a flask. To this, 0.3ml of 5% $NaNO_2$ was added and at the end of 5min incubation 0.3ml of 10%$AlCl_3$ was added and re-incubated for another one minute. Finally, 2ml of 1M NaOH was added and then the contents were made up to 10ml with distilled water. All the contents were mixed and the absorbance was measured at 510nm. The standard curve for total flavonoids was made using rutin (0 to 100 µg/ml) in methanol. . Results were expressed as mg of rutin equivalent /g of dry sample.

DETERMINATION OF TANNIN CONTENT

Tannin content in the extracts was estimated by Folin-Denis method [10] with tannic acid as standard solution at a concentration of 100µg/ml. One ml (10mg/1ml) of the individual ex-

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tract was mixed with 0.5ml of Folin-Denis reagent and 1ml of saturated carbonate solution. The contents were vortexed and allowed it to stand for 30 min and the intensity of the color was read at 760nm against reagent blank. All the values were determined in triplicates and the mean was calculated.

DETERMINATION OF ANTIOXIDANT ACTIVITY BY FRAP METHOD

The FRAP assay was carried out by Benzie and Strain method with some modifications [11]. 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 prepared freshly by mixing 25ml of acetate buffer (Ph 3.6) with 2.5 ml of 10 mM 2,4,6 tripyridyl triazine (TPTZ) and 2.5 ml of 20mM Ferric chloride solution. The reagent was kept at 37 °C before use.

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

DETERMINATION OF ANTIBACTERIAL ACTIVITY

Antibacterial activity of all the extracts prepared in different solvents from dried fruit sample of AO was determined using standard agar well diffusion method [12]. The bacterial strains used in our study were Enterobacter aerogenes MTCC 7324, Enterobacter cloacae MTCC 7408, Staphylococcus aureus MTCC 737 and Streptococcus pyogenes MTCC 1928.. Each experiment was performed in triplicates and the average value for zone of inhibition was calculated. The zones were compared with that of broad spectrum antibiotic Gentamicin 30µg/disc [13].

DETERMINATION OF MIC

Minimum Inhibitory Concentration [MIC] was determined by broth dilution assay method [14]. 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

The reports of flavonoid and tannin are given in table 1. From this data it is very clear that flavonoids and tannins are there in both the solvents. Comparatively, flavonoids content is high than tannin in both the solvents employed. These secondary metabolites are incredible group of compounds present in all the plants. Ethyl acetate is considered to be the best solvent for the extraction of flavonoid. Whereas, methanol is consider to be best suited for tannin purification.

Table. 1. Flavonoid and Tannin content in root extracts of Derris trifoliata

	Flavonoid mg RE/ g Extract	Tannin mg/g Extract
Ethyl acetate	57.66	13.66
Methanol	36.33	28.33

Many plants have been investigated for the antioxidant activities and the search is gradually increased in recent times. Several techniques have been used to determine the antioxidant activity. Antioxidants fight against free radicals and protect us from various diseases. The antioxidant activity of the ethyl acetate and methanol infusion are estimated spectrophotmetrically by FRAP method and the data is given in table-2. The outcome of antioxidant activity is high in methanol extract than ethyl acetate. These results correlate with the presence of flavonoid and tannin in roots of Derris trifoliate

Table-2. Antioxidant activity of root extracts of Derris trifoliata by FRAP method.

	Frap units µ mol/g Extract
Ethyl acetate	438
Methanol	864

ANTIBACTERIAL ACTIVITY

In vitro antibacterial activity of root extracts of Derristri foliata was determined by agar well diffusion method. This experimental data is presented in Figure-1. The ethyl acetate and methanol infusions were found to possess various degrees of antibacterial activity against both Gram positive and Gram negative bacteria. Among the tested extracts methanol solubles of Derristri foliata exhibited highest potential of antibacterial activity (12.66 mm to 17.66 mm) against all the tested bacterial species irrespective of their Gram nature. However, components of Derristri foliata infused in ethyl acetate were found to be active against Enterobacter aerogenes and Enterobacter cloacae with a zone size of 16.33 mm. However, the principle molecules present in ethyl acetate extracts inhibited only the growth of Streptococcus pyogenes but not Staphylococcus aureus. None of the extracts will have higher zone of inhibition than gentamicin.

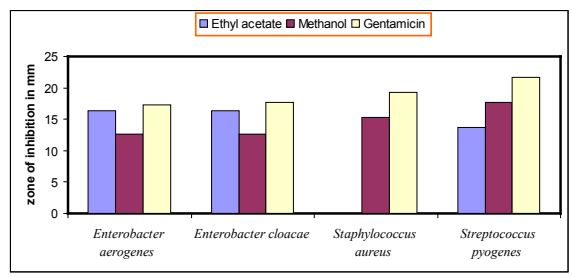

Fig-2 Antibacterial activity of Derris trifoliata against the test cultures.

Further, these extracts were analyzed to determine the minimum inhibitory concentration. The MIC values ranged from 2.5-5mg/100µl and varied from one extract to the other and the results are displayed in table-3. The MIC of methanol extracts towards the gram negative tested cultures is 5mg/100µl. Where as, for gram positive test culture it is 2.5mg/100µl . However, the infusion of ethyl acetate are having lower MIC values (2.5 mg/100µl) to gram negative cultures used than gram positive microorganism.

Table-3: MIC of Derris trifoliata root extracts (mg/100µl)

Micoorganisms	Ethyl Acetate	Methanol
Enterobacter aerogenes	2.5	5
Enterobacter cloacae	2.5	5
Enterococcus faecalis	-	2.5
Streptococcus pyogenes	5	2.5

DISCUSSION

Natural products, such as plant extract, either as pure compounds or standardized extracts provide infinite opportunities for new drug discoveries. Mangroves are commonly available plant in almost all the coastal states of India and are diverse group of plants rich with many secondary metabolites like alkaloids, flavonoids, phenolics, steroids, tannins and terpenoids These secondary metabolites are believed to account mainly the antioxidant activity of plants [15, 16]. Tannins exhibit many biologically important functions, such as protection against oxidative stress, and degenerative diseases. Flavonoids are a group of naturally occurring polyphenolic compounds ubiquitously found in plants and have long been recognized to possess anti-inflammatory, antioxidant, ant-allergic and hepato-protective properties. They are also believed to be antibacterial, antifungal, antiviral, and cancer protective, and also to protect against cardiovascular disease [17- 19]. In folk medicine mangrove plant parts and extracts are being used in the treatment of various

diseases over the centuries. In this study, we have extracted the flavonoid and tannin by standard protocols, quantitatively determined the amount of flavonoids and tannin so also the antioxidant and antibacterial potentials of Derris trifoliata root constituents in ethyl acetate, and methanol . Our results show that the secondary metabolites present in the root extracts of Derris trifoliate are more of polar in nature as their content is observed in ethyl acetate and methanol solvents used. The antioxidant activity is high for methanol extract and it is correlated with the phytochemical studies. The present experimental data is in accordance with that of Govindasamy et.al [20] they 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. These are also known to be associated with other biological activities such as antibacterial, antifungal, antidiuretic activities [21]. In search of potent broad spectrum antibacterial compounds, in this study we have screened for inhibitory effect of Derris trfolitata root extracts in ethyl acetate and methanol against two gram negative and two gram positive test cultures, viz., Enterobacter aerogenes Enterobacter cloacae, Staphylococcus aureus and Streptococcus pyogenes. The tested root extracts of Derris trfolitata possessed different levels of antibacterial activities. The activity of methanol extracts on the bacterial test cultures confers their broad spectrum nature over the ethyl acetate extract. The zone of inhibition exhibited by the methanol extracts were higher towards gram positive test cultures than gram negative cultures. The difference in rate of inhibition activities appear to be directly related to the quantitative diversity of the compounds that are present in the extract. This may be due to the permeability factor of cell membrane of the microorganism [22], 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 [23]. On correlating our results of secondary metabolites and antibacterial activities it is inferred that flavonoids, and tannins possess substantial antibacterial activity. Ethyl acetate extract did not inhibit Staphylococcus aureus however, 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 have mechanism to over come the effect of the bioactive present in the extract. In our study, the MIC value for all the positive extracts against the tested bacteria were between 2.5mg/100µl to 5mg/100µl. Gram negative test cultures showed higher MIC values than Gram positive text cultures to the root extracts in methanol.

This difference may be explained by susceptibility testing condition, physico 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[24,25]. But this can be substantiated by the argument that this value is for the crude extract. However, the purified form of bioactive compound of the crude extract responsible for antibacterial activity may exhibit the inhibitory effect at a lower concentration.

Fai-Chu Wong [26] 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.

Hence Derris trifoliate is strongly recommended for considering a valuable source for isolation, identification and characterization of bioactive principle responsible for antioxidant and antibacterial activity. However, further work in this direction could lead to the discovery of powerful bioactive principle from the Derris trifoliata

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