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Extraction of Secondary Metabolites from Roots of Acanthus Ilicifolius L and Screening for Antioxidant and Antibacterial Activity.

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Abstract: *The root extracts of Acanthus ilicifolius L finds a prominent place in folk medicine. In this study, we extracted alkaloid, flavonoid, tannin and total phenols in benzene, ethyl acetate, acetone, methanol and ethanol, their antibacterial activity and antioxidant activity was evaluated. The antioxidant activity is executed by FRAP assay and agar well diffusion method is done to study the antibacterial activity against Enterobacter aerogenes, Enterobacter cloacae, Escherichia coli, Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogenes. The antibacterial activity of all the extracts was compared with standard antibiotic gentamicin. The Minimum Inhibitory Concentration [MIC] was determined by serial dilution method. Alkaloids are rich in acetone and Flavonoids are high in methanol extracts. The acetone extract showed higher antioxidant activity, while benzene extract was identified to contain lower antioxidant activity. The extent of inhibition by the root extracts diverge between the solvents used, among them ethanol extracts exhibited higher level of inhibition against the gram positive test cultures compared to gram negative test cultures employed. Whereas, the acetone extracts efficacy is more on gram negative test cultures than the gram positive cultures. The MIC was found to be between 1mg/100µl to 5mg/100µl. This study gives the source for purification and characterization of bioactive principles that possess antioxidant and antibacterial action from the root of Acanthus ilicifolius.*
Keywords: *Acanthus ilicifolius, Root, Antibacterial activity, Antioxidant activity*

I. 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. Mangroves are diversified group of plants that grow in estuarine environment, have been a source of interest for their novel natural products like alkaloids, flavanoids, glycosides, saponins, tannins, etc., are known to exhibit antiviral, antibacterial and antifungal activities [1] Systematic study of mangrove species has revealed that crude extracts of different plant parts in different solvents exerted potential antibacterial, antifungal, antiviral and antioxidant activities [2]. Therefore, the present study has designed to extract the secondary metabolites and evaluate their antibacterial and antioxidant activity from the roots of *Acanthus ilicifolius*.

II. Materials and Methods

Collection of Plant material

Acanthus ilicifolius belongs to the family *Acanthaceae* also be called as Holly Mangrove, Holly-leaved Acanthus. The plant is used in traditional systems of medicine, including Traditional Indian Medicine (TIM) and Traditional Chinese Medicine (TCM). The plant part were collected from Corangi Reserved Forest, Kakinada, East Godavari, Andhra Pradesh, India. Geographic location - between 16^o 39' N longitude - 17^o N longitude and 82^o 14' E latitude - 82^o 23'E latitude. All the roots were surface sterilized with 1% mercuric chloride solution and thoroughly washed with filter sterilized distilled water [3]. 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 benzene, ethyl acetate, acetone methanol and ethanol were prepared according to the standard protocols [4]. The chopped root material (1000g) was initially soaked in 1000ml 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 temperatures using vacuum rotator evaporator. These extracts were further concentrated by solvent evaporation using thin film method.

Identification of Secondary Metabolites.
The crude extracts were subjected to the qualitative determination of secondary metabolites viz., alkaloids, flavonoids, glycosides, tannins and total phenols by the standard protocols of phytochemistry [5]

Test for Alkaloids
To 5 ml of the crude extracts were stirred with 10 ml of 1% aqueous HCl on water bath and then filtered. To 2ml filtrate 4-6 drops drops of Dragendroff's reagent was added. Formation of orange –red precipitate was considered as positive to alkaloids.
To another 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. Then, 2 ml of lead acetate solution was added to 2 ml of the filtrate. Appearance of buff coloured precipitates considered as presence of 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 tannins
About 5ml of each extract was stirred with about 10ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2ml of the filtrate. Occurence 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.

Tests for total phenols
Five ml of the extract was dissolved in 5 mL of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. Appearance of dark green color indicates the presence of phenolic compounds.
Five ml of the extract was dissolved in 5 mL of distilled water. To this, 3ml of 10% lead acetate was added. Formation of bulky white precipitate indicate the presence of phenol compounds.

Determination of antioxidant activity by ABTS method
Total antioxidant capacity of each extract was measured using 2, 2´-azinobis [3-ethylbenzthiazoline]-6-sulfonic acid (ABTS) assay. ABTS and potassium per-sulfate were separately dissolved in deionized distilled water to a final concentration of 7mM and 2.45mM respectively. The two solutions were mixed and allowed to stand in dark at room temperature for 16h before use in order to produce ABTS radical (ABTS•+). The resultant intensely-coloured ABTS•+ radical cation was diluted with 0.01M PBS (phosphate buffered saline), pH 7.4, to give an absorbance value of ~0.70 at 734 nm. The test compound was diluted 100X with the ABTS solution to a total volume of 1ml. Absorbance was measured spectrophotometrically at time intervals of 3min after addition of each extract. The assay was performed at least in triplicate. Controls were run using PBS in place of the extract. The assay relies on the antioxidant capability of the samples to inhibit the oxidation of ABTS to ABTS•+ radical cation. Percent inhibition was calculated using the following formula and it was compared with ascorbic acid. [6]

$$\% \text{ inhibition of oxidation of ABTS to ABTS}^{\bullet+} = \frac{\text{Initial absorbance - Final absorbance X 100}}{\text{Initial absorbance}}$$

Determination of antibacterial activity
The antibacterial activity of the crude extracts was screened with gram negative and gram positive cultures viz., *Enterobacter aerogenes*, *Enterobacter cloacae*, *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Streptococcus pyogenes*. by agar well diffusion method [7]. About 20 ml of melted Mueller Hinton agar was mixed with 1 ml of bacterial suspension homogeneously and allowed to solidify in petri dishes (143 mm diameter). Wells (8mm diameter) were made using a sterile cork borer on the solidified medium and are filled with 100 µl of the extract (5mg/100µl). The inoculated plates were incubated at 34-38^{o}C. The diameter of the inhibition zones were measured in mm. Each experiment was performed in triplicate and the average value of inhibition and standard deviation was calculated. Gentamicin (30µg) discs are used as positive control and DMSO with solvents (1:10 dilution) used as control. Results were expressed as mean ± SD and the data were analyzed using one-way analysis of variance (ANOVA) to discover the significant difference at the 5% ($P<0.05$)level.

Determination of MIC

Minimum Inhibitory Concentration [MIC] was determined by broth dilution assay method [8]. Plant extracts were serially diluted in Mueller Hinton broth to get the concentrations of 1.0 to 5.0 mg/100µl. Each experiment was repeated thrice and the mean values were tabulated.

III. Results

Plants are the important source for the development of new bio active principles, the knowledge of secondary metabolites and their biological properties are desirable, not only for the discovery of novel bioactive entities, but also for disclosing new sources of already known biologically active compounds. *Acanthus ilicifolius* is a medicinal plant used against asthma, paralysis, snake bite, analgesic and anti inflammatory. The reports of phytochemical studies are given in table 1. From this data it is very clear that tannins and total phenols are there in all the solvents used for the extraction of secondary metabolites. However, flavonoids are seen in all the solvents except benzene. Whereas, alkaloid is confined only to acetone, methanol and ethanol. These secondary metabolites are incredible group of compounds plays an important role in many biological activities.

The antibacterial activity of root extracts of *Acanthus ilicifolius* in benzene, ethylacetate, acetone, methanol and ethanol was revealed by agar well diffusion method and the results are presented in table-2. All the extracts have assorted degrees of antibacterial potential against cultures used. Among the tested extracts benzene solubles of *Acanthus ilicifolius* exhibited highest potential of antibacterial activity (19.66mm) against *Enterobacter cloacae* than gentamicin. The ethyl acetate soluble fraction is active against *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* with same zone size of 16.66 mm. However, metabolites of *Acanthus ilicifolius* infused into acetone were found to be active on selective test cultures viz., *Enterobacter aerogene* and *Enterobacter cloaceae* of gram negative and *Staphylococcus aureus* of gram positive bacteria. The infusions of ethanol are susceptible to all the test cultures used except *Enterobacter aerogenes* and the inhibitory effect against test cultures is in increasing order for *Enterobacter cloacae* (12.66), *Escherichia coli* (13.33) *Streptococcus pyogenes* (17.66), *Bacillus subtilis* (18.33)and *Staphylococcus aureus* (18.66). Whereas, the efficacy of methanol extracts are sensitive to *Enterobacter aerogenes* and *Escherichia coli* with same zone size of 16.66 mm and zone size of 17.33 was observed with *Bacillus subtilis* and *Staphylococcus aureus*. The maximum zone of inhibition exerted against gram negative test cultures is *Enterobacter cloacae* (ethylacetate extract) 19.66 mm and gram positive is *Staphylococcus aureus* (ethanol extract) 18.66 mm. The zone of inhibition exerted by gentamicin is same for all the gram positive test cultures employed. Whereas, among gram negative *Enterobacter aerogenes* and *Enterobacter cloacae* are less sensitive then *Escherichia coli* to gentamicin. The inhibitory effect of the root extracts of *Acanthus ilicifolius* in ethyl acetate and ethanol against the test cultures is inferior than gentamicin. However the inhibitory outcome of acetone extracts were superior than gentamicin against the two gram negative cultures used viz., *Enterobacter cloacae* and *Escherichia coli*. Whereas, the infusions of methanol having higher inhibitory potential than gentamicin against *Enterobacter aerogenes* and *Enterobacter cloacae*. We also studied the combination of different extracts and the results are displayed in table-3. The effect of ethyl acetate and acetone extract mixture have mixed result it has antagonistic effect on *Bacillus subtilis* and symbiotic effect on *Escherichia coli*. The mixture of ethyl acetate and methanol have not net role on gram negative cultures used but have negative effect on the gram positive cultures employed in terms of inhibition zone size. Ethyl acetate and ethanol mixture did not inhibit the *Enterobacter aerogenes, Enterobacter cloacae* and *Streptococcus pyogenes*. But the zone of inhibition against gram positive test organism viz., on *Bacillus subtilis* and *Staphylococcus aureus* is very high compare to that of the individual extracts and the phenomenon is called symbiotic effect. The combination of acetone and methanol extracts is the best as they control the growth of all the test organisms employed in our study. Acetone and ethanol mixture has shows antagonistic effect with respect to inhibition. Methanol and ethanol mixture have antagonistic effect on *Enterobacter cloacae* and *Staphylococcus aureus*.

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 1.0-5.0 mg/100µl and varied from extract to extract and the results are given in table-3. The MIC of root extracts in benzene is 2.0 mg/100µl against *Enterobacter cloacae*. Ethyl acetate extracts were active at 3.0 mg/100µl with *Escherichia coli* and 2.0 mg/100µl with *Bacillus subtilis* and *Staphylococcus aureus* . Whereas, the MIC of acetone extract is 2.0 mg/100µl towards all the tested microorganisms. The methanol extracts of *Acanthus ilicifolius* root exhibiting diversified range of MIC among the tested cultures between 1.0 - 4.0 mg/100µl. Whereas, the MIC of ethanol extract is 5.0 mg/100µl against gram negative test cultures and 3.0 mg/100µl with gram positive microorganisms used.

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 symptoms. Antioxidants fight against free radicals and protect us from various diseases. The non enzymatic antioxidant activity of plants is attributed to the secondary

metabolites. The antioxidant activity of the fractions was estimated spectrophotmetrically by ABTS method and the data is given in Fig-1. The outcome of antioxidant activity by ABTS method is high for acetone extract followed by ethanol, methanol ethyl acetate and benzene extracts.

IV. Discussion

In this study, we have qualitatively screened, specifically for the presence of alkaloids, flavonoids, tannin and total phenols as well as the antioxidant and antibacterial potentials of *Acanthus ilicifolius* root infusions in benzene, ethyl acetate, acetone, methanol and ethanol in order of increasing polarity. Alkaloids, flavonoids, tannin and total phenols are associated with potent biological activities. Our results show that the secondary metabolites present in the root extracts of *Acanthus ilicifolius* are diversified in the solvents used. Acetone, methanol and ethanol fractions contain all the secondary metabolites checked. Our observation are in accordance with the report of Asha *et.al*, they extracted flavnoids and phenolics from the root extracts of *Acanthus ilicifolius* in ethanol [9]. The efficacy of antibacterial action is high for ethanol extract and it correlates with the presence of all the secondary metabolites studied. The present experimental data is in accordance with that of Govindasamy *et.al* who studied the chemical constituents of different mangroves in India [10]. The secondary metabolites viz., alkaloids, flavonoids tannin and total phenols play an important role in scavenging of free radical and are also known to be associated with other biological activities such as antibacterial, antifungal, antidiuretic activities [11,12]. The root extracts of in ethyl acetate, methanol and ethanol are active against *Escherichia coli Bacillus subtilis* and *Staphylococcus aureus* these results are in disagreement with reports of dev avijit *et.al*[13]. They studied the stem extract of *Acanthus ilicifolius* in methanol by maceration and continuous extraction method. The difference of the result may be due to the plant part used or the protocol employed for the extraction.

The extracts in ethyl acetate were active against *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus* with same zone size of 16.66 mm. This may be the compounds present in the extract may have the similar effect on these organisms. The activity of ethanol extracts on all the bacterial cultures except *Enterobacter aerogenes* confers their broad spectrum nature over the other extracts. The zone of inhibition exhibited by the acetone extracts were higher towards gram negative organisms than gram positive organisms and the ethanol extracts were more active on gram positive than gram negative cultures. This difference in the rate of inhibition potential appear to be directly related to the qualitative diversity of the compounds that are present in the extracts. This may be due to the permeability factor of cell membrane of the microorganism, or this could be due to variation in the cell wall composition of Gram negative and Gram positive bacteria[14]. 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. On correlating our results of secondary metabolites and antibacterial activities it is inferred that alkaloids, flavonoids, glycosides tannins and total phenol possess substantial antibacterial activity. Our results are in concurrence with the work of Bose and Bose [15], with reference to the solvent system and type of microorganisms, i.e., gram positive cultures are more sensitive than gram negative cultures used.

The more effectiveness of root extracts of *Acanthus ilicifolius* in methanol and ethanol than that of the acetone, benzene and ethyl acetate in our study can be correlated with the medicinal preparation that use rum and liquour to extract the active plant components. Benzene extracts did not inhibit *Enterobacter aerogenes, Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and *Streptococcus pyogenes*. Similarly ethyl acetate infusions also did not inhibit *Enterobacter aerogenes, Enterobacter cloacae*, and *Streptococcus pyogenes*. Negative results did not mean the absence of bioactive principles in these extracts. The bioactive principle may be insufficient to cross the membrane, or the microorganism may have mechanism to nullify the effect of the bioactive principle present in these extracts.

In our study, the MIC value for all the positive extracts against the tested bacteria were between 1.0mg/100µl to 5mg/100µl. Gram negative test cultures showed higher MIC values than Gram positive text cultures to the 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 [16]. 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[17] 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 *Acanthus ilicifolius* is strongly recommended for considering a valuable source for isolation, identification and characterization of bioactive principle responsible for antibacterial activity on various bacteria and fungi. Also there is a need to study the different plant parts of *Acanthus ilicifolius* in other organic solvents apart from the solvents employed in our study. However, further work in this direction could lead to the discovery of powerful bioactive principles from the *Acanthus ilicifolius*

Table-1 Secondary metabolites of root extracts of *Acanthus ilicifolius*

	Alkaloid	Flavonoid	Tannin	Total Phenols
Benzene	-	-	+	+
Ethyl acetate	-	+	+	+
Acetone	+	+	+	+
Methanol	+	+	+	+
Ethanol	+	+	+	+

+ = Present - = Absent

Table-2 Antibacterial activity of *Acanthus ilicifolius* root extracts

	Benzene	Ethylacetate	Acetone	Methanol	Ethanol	Gentamicin
Enterobacter aerogenes	-	-	-	16.66±0.57	-	15.33±0.57
Enterobacter cloacae	19.66±0.57	-	17.33±0.57	-	12.66±1.52	15.33±0.57
Escherichia coli	-	16.66±0.57	16.66±0.57	16.66±0.57	13.33±0.57	17.33±0.57
Bacillus subtilis	-	16.66±1.15	-	17.33±0.57	18.33±1.15	18.66±0.57
Staphylococcus aureus	-	16.66±1.52	14.33±0.57	17.33±0.57	18.66±0.57	18.66±0.57
Streptococcus pyogenes	-	-	-	-	17.66±1.52	18.66±0.57

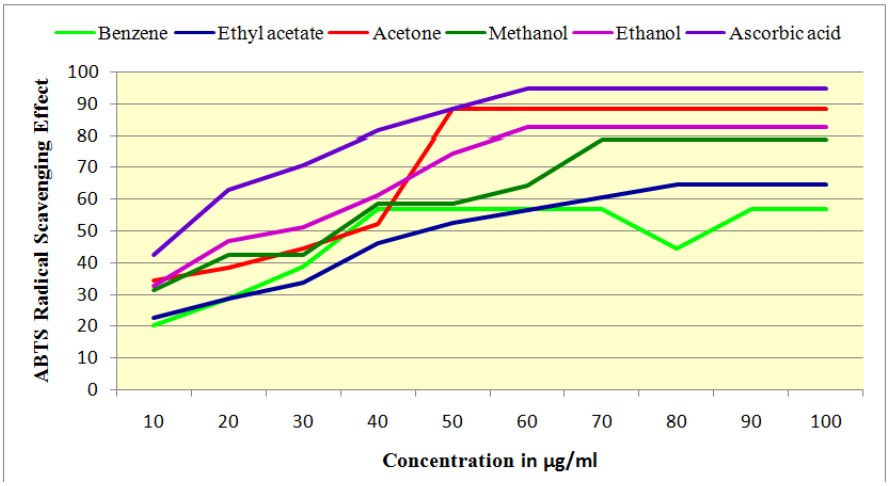
Table-3 Antibacterial activity of *Acanthus ilicifolius* root extract Mixture

	Ethylacetate + Acetone	Ethylacetate + Methanol	Ethylacetate + Ethanol	Acetone + Methanol	Acetone + Ethanol	Methanol + Ethanol
Enterobacter aerogenes	-	16.33±0.57	-	16.66±0.57	-	15.33±0.57
Enterobacter cloacae	16.33±0.57	-	-	16.33±1.15	-	
Escherichia coli	18.66±0.57	16.66±0.57	16.66±0.57	16.66±0.57	14.66±1.15	17.33±0.57
Bacillus subtilis	-	13.66±1.15	19.66±1.15	15.33±0.57	19.33±1.15	18.66±0.57
Staphylococcus aureus	14.66±0.57	14.66±1.52	19.66±1.52	15.33±1.52	18.66±0.57	-
Streptococcus pyogenes	-	16.66±0.57	-	17.66±1.52	17.66±1.52	18.66±0.57

Table-4 MIC of *Acanthus ilicifolius* root extracts (mg/100µl)

	Benzene	Ethylacetate	Acetone	Methanol	Ethanol
Enterobacter aerogenes	-	-	-	4.0	-
Enterobacter cloacae	2.0	-	2.0	-	5.0
Escherichia coli	-	3.0	2.0	2.0	5.0
Bacillus subtilis	-	2.0	-	3.0	3.0
Staphylococcus aureus	-	2.0	2.0	1.0	3.0
Streptococcus pyogenes	-	-	-	-	3.0

Fig-1. Antioxidant activity of *Acanthus ilicifolius* by ABTS method.



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