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ANTIBACTERIAL AND PHYTOCHEMICAL STUDY OF *ACANTHUS ILICIFOLIUS* L. STEM EXTRACTS

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ABSTRACT

Objectives: To investigate the phytochemical and antibacterial activity of the stem infusions of *Acanthus ilicifolius* L. **Methods:** Qualitative determination of alkaloids, flavonoids, glycosides, tannins and total phenols in different solvent extracts was performed by the standard protocols. Antibacterial activity of all the extracts was screened against the selected strains by agar well diffusion method and compared with standard antibiotics gentamicin and tetracyclin. The minimum inhibitory concentration [MIC] was determined by serial dilution method. **Results:** Phytochemical screening revealed that the stem infusions were found to be positive for alkaloids, flavonoids, glycosides, tannins and total phenols. The inter-solvent differences were observed in the extent of inhibition by the stem extracts. Methanol and ethanol extracts exhibited higher level of inhibition for the gram positive than the gram negative test cultures employed. The value of MIC was found to be between 2-7mg/100 µl. **Conclusion:** Isolation and characterization of antibacterial bioactive principles from stem extracts of *Acanthus ilicifolius* was established.

KEYWORDS: *Acanthus ilicifolius*, Secondary metabolites, Antibacterial activity, Minimum Inhibitory concentration.

INTRODUCTION

The wide array of plant bioactive molecules is rich source of medicines. The use of plant extracts and phytochemicals, with known antibacterial properties, may be of immense importance in therapeutic treatments. About 80% populations of the developed countries use traditional drugs, derived from medicinal plants. A large number of plant infusions helped in

combatting diseases and are known to possess antimicrobial activity.[1,2] The screening of indigenous plants for antimicrobial properties may yield useful results. Plant based drugs are implicated in less/no side effect(s) compared to synthetic antibiotics.[3] Recent years have witnessed an alarming rate of increase in antibiotic resistant microorganisms. This has brought exploration of plant derived antimicrobial drugs to the vanguard.[4] Mangroves are perennial plants that grow in coastal wetlands of tropical regions. These are source of 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] *Acanthus ilicifolius* L belongs to the family acanthiaceae. In the present study, we examined different extracts prepared from the stem of *Acanthus ilicifolius* for their secondary metabolites as well as antibacterial activity.

MATERIALS AND METHODS

Collection of Plant material

The plant materials (stem specimen) were collected from Corangi reserved forest, Kakinada, East Godavari, Andhra Pradesh, India. The place is geographically located between 16^{o} 39'-17^{o} N longitude and 82^{o} 14'-82^{o} 23'E latitude. The stem specimens were surface sterilized with 1% mercuric chloride solution and thoroughly washed with filter sterilized distilled water.[7] The washed stem, 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 protocols.[8] The chopped stem material (1000g) was initially soaked in 2000ml 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 filter paper, Whatman number-1 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. Dried stem 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.[9] All the extracts thus prepared were stored in a refrigerator at 4^{o}C.

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.[10,11]

Test for Alkaloids

Crude extracts (5 ml) were stirred with 10ml of 1% aqueous HCl on 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 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 indicated presence of flavonoids. To 2 ml of the filtrate, 5 ml of dilute ammonia solution was added followed by 4 – 6 drops of concentrated sulphuric acid. Appearance of yellow color indicated the presence of flavonoids.

Test for Glycosides

To 2 ml of each extracts, 1 ml of pyridine, 1 ml of Sodium nitro-prusside solution were added. Appearance of pink or red color indicated the presence of glycosides. To 2ml of each extract, 2 ml of glacial acetic acid and 1 ml of $FeCl_3$ solution were added. The resultant solution was heated and then cooled. This was transferred to a test tube containing 2ml conc. H_2SO_4 and observed for reddish brown ring at the interface.

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. The appearance of a blue-black, green or blue-green precipitate indicated the presence of tannins. About 5 ml of each extract was added with 1 ml of 1% HCl solution. Formation of red precipitate indicated the presence of tannins.

Test 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 indicated 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 indicated the presence of phenol compounds.

Determination of Antibacterial Activity

Antibacterial activity of all the extracts prepared in different solvents from dried stem sample of *Acanthus ilicifolius* was determined using standard agar well diffusion method.[12,13,14] The bacterial strains *Escherichia coli* MTCC 7410, *Enterobacter aerogenes* MTCC 7324, *Enterobacter cloacae* MTCC 7408, *Klebsiella pneumonia* MTCC 7028, *Bacillus subtilis* MTCC 736, *Enterococcus faecalis* MTCC 9845, *Staphylococcus aureus* MTCC 737 and *Streptococcus pyogenes* MTCC 1928 were used. 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 and Tetracyclin with a concentration of 10µg/disc.[15] Results were expressed as mean±SD and the data were analyzed using one-way analysis of variance (ANOVA) to discover the significant difference at 5% ($p<0.05$) level.[16]

Determination of MIC

Minimum Inhibitory Concentration [MIC] was determined by broth dilution assay method.[17] Plant extracts were serially diluted in Mueller Hinton broth to get the concentrations of 1 to 10mg/100µl. Each experiment was repeated thrice and the mean values were tabulated.

Determination of Bactericidal (BC)/Bacteriostatic activity (BS)

Bactericidal and/or bacteriostatic activity was determined by Damintoti *et al* method.[18] Exactly, 0.1 ml of culture medium was aspirated from each broth assay tube showing no apparent growth and sub-cultured on fresh Mueller Hinton agar medium. After incubation at 37^{o}C for 24 hrs, plates showing no visible growth of bacteria were considered for bactericidal effect and plates with visible growth of bacteria as bacteriostatic.

Determination of susceptibility factor of microorganism and inhibitory potential of the chemicals were deduced as follows.

$$\text{Susceptibility factor} = \frac{\text{Number of microorganisms sensitive to extracts}}{\text{Total number of microorganisms}} \times 100$$

$$\text{Inhibitory Potential} = \frac{\text{Number of microorganisms sensitive to extracts}}{\text{Total number of extracts}} \times 100$$

RESULTS

The record of phytochemical studies is given in Table 1. Flavonoids and total phenols were present in all the solvents used for the extraction of secondary metabolites. Alkaloids were found in all the solvents but ethyl acetate. Glycosides and tannins were observed only in the alcoholic solvents.

The results of the in vitro antibacterial activity of all the stem extracts of *Acanthus ilicifolius* performed by agar well diffusion method are presented in Figure-1. All the extracts were found to possess different level of antibacterial activity against both Gram negative and Gram positive bacteria used. Methanol solubles of *Acanthus ilicifolius* exhibited highest potential of antibacterial activity (12.66mm to 19.33mm) against all the tested bacterial species. However, ethyl acetate infusions were found to be selectively active viz., *Enterobacter aerogene* and *Enterobacter cloaceae* of gram negative with zone size of 16.33mm and *Bacillus subtilis* and *Enterococcus faecalis* of gram positive with 13.66 mm zone size. The acetone soluble fraction was active against all the test cultures used except *Enterobacter cloacae* and *Staphylococcus aureus*.

The infusions of methanol were susceptible to all the test cultures used and the inhibitory effect against test cultures is in increasing order for *Enterobacter cloacae* (12.66), *Enterobacter aerogenes* (14.66), *Klebsiella pneumonia* (15.33), *Enterococcus faecalis* and *Streptococcus pyogenes* (16.66), *Escherichia coli* (18.66) and *Bacillus subtilis* and *Staphylococcus aureus* (19.33). Ethanol extracts were sensitive to all the extracts except *Enterococcus faecalis*. The zone of inhibition exerted against gram negative test cultures used were higher compare to that of the gram positive test cultures. The maximum zone of inhibition for *Escherichia coli* was (19.66) mm.

The inhibitory power of gentamicin is more effective than tetracylin against *Enterobacter cloacae*, *Klebsiella pneumonia*, *Bacillus subtilis*, *Enterococcus faecalis* and *Streptococcus*

pyogenes. The zone of inhibition for gentamicin and tetracycline is same for *Staphylococcus aureus*. Whereas, *Enterobacter aerogenes* and *Escherichia coli*, sensitivity is slightly higher for tetracycline then gentamicin. The stem extracts of *Acanthus ilicifolius* in ethyl acetate and ethanol exhibited more inhibitory effect than gentamicin and tetracycline against *Enterobacter aerogenes* and *Enterobacter cloacae*. The inhibitory effects of acetone extracts were inferior to the two antibiotics employed. The infusions of methanol had higher inhibitory potential than the two antibiotics used against *Enterobacter cloacae, Bacillus subtilis* and *Staphylococcus aureus*. The inhibitory effect of the ethanol extracts on all gram negative test cultures were superior than that of the gentamicin and tetracyclin but inferior for gram positive test cultures.

The MIC values showed inter-culture and inter-solvent variations (Table-2). The acetone infusions had a wide range of MIC (2.0-7mg/100µl) values. The MIC of methanol extracts was 4mg/100µl against gram negative test cultures and 2mg/100µl for gram positive bacterial cultures used. The MIC of ethanol extract was 3mg/100µl for gram negative microorganisms and 5mg/100µl for *Staphylococcus aureus* and *Streptococcus pyogenes*. Ethyl acetate extracts were active at 7mg/100µl against *Enterobacter aerogenes, Enterobacter cloacae, Bacillus subtilis* and *Enterococcus faecalis*.

The bactericidal/bacteriostatic activity results are given in Table-3. Methanol extracts were bactericidal and ethyl acetate extracts were bacteriostatic against cultures used. Ethanol extracts were bacteriostatic with gram negative cultures and bactericidal with gram positive bacterial test cultures. However, the infusions of acetone exhibited mixed result. The data of susceptibility studies is given in fig-2 and it reveals that *Enterobacter aerogenes* and *Bacillus subtilis* are most sensitive to the infusions of *Acanthus ilicifolius* and *Staphylococcus aureus* is less sensitive. Fig-3 disclose that inhibitory potential is high for stem infusions of *Acanthus ilicifolius* in methanol followed by ethanol it correlates with the presence of secondary metabolites observed compare to that of the other solvents.

Table-1. Phytochemical analysis of stem extracts of *Acanthus ilicifolius*

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

+ = Present - = Absent.

Table-2: MIC of *Acanthus ilicifolius* stem extracts (mg/100µl)

Microorganism	Ethyl acetate	Acetone	Methanol	Ethanol
Enterobacter aerogenes	7	5	4	3
Enterobacter cloacae	7	-	4	3
Escherichia coli	-	3	4	3
Klebsiella pneumonia	-	6	4	3
Bacillus subtilis	4	4	2	5
Enterococcus faecalis	4	7	2	-
Staphylococcus aureus	-	-	2	5
Streptococcus pyogenes	-	2	2	5

Table-3: BC/BS Activity of *Acanthus ilicifolius* stem extracts

Microorganism	Ethyl acetate	Acetone	Methanol	Ethanol
Enterobacter aerogenes	BS	BS	BC	BS
Enterobacter cloacae	BS	-	BC	BS
Escherichia coli	-	BC	BC	BS
Klebsiella pneumonia	-	BS	BC	BS
Bacillus subtilis	BS	BC	BC	BC
Enterococcus faecalis	BS	BS	BC	-
Staphylococcus aureus	-	-	BC	BC
Streptococcus pyogenes	-	BC	BC	BC

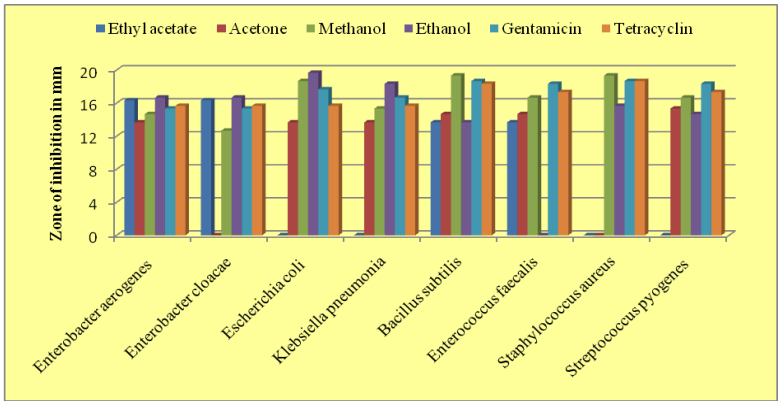

Fig-1 Antibacterial activity of *Acanthus ilicifolius* against the test organisms.

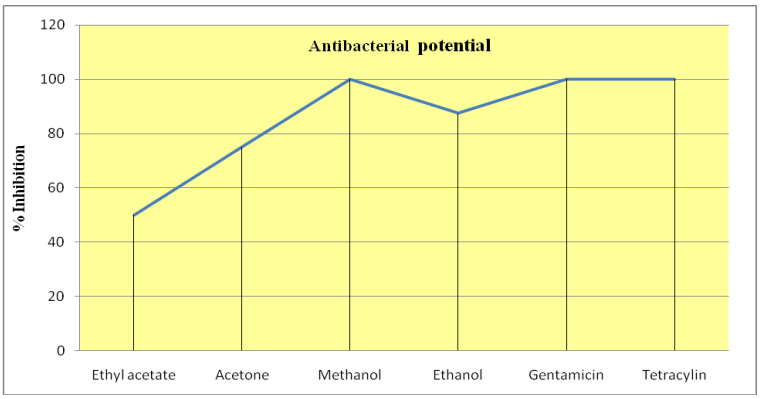

Fig-2. Antibacterial potential of Test (Extracts) and Standard (Antibiotics)

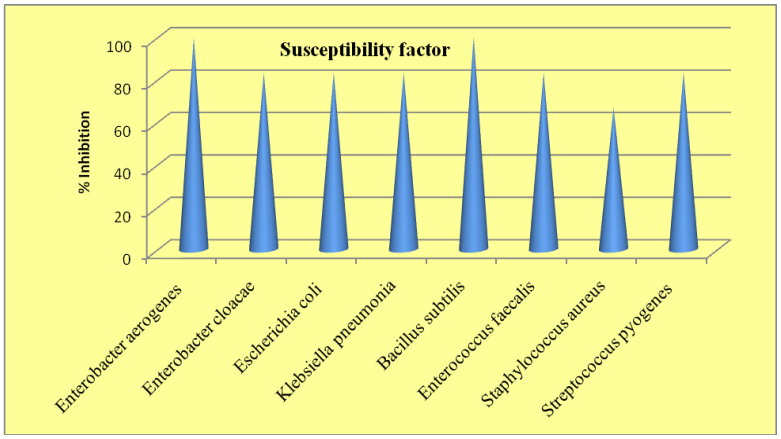

Fig-3. Susceptibility of microorganisms to Tests (Extracts) and Standard (Antibiotics)

DISCUSSION

Our results show that the secondary metabolites present in the stem extracts of *Acanthus ilicifolius* are polar in nature. This was indicated by the presence of types of secondary metabolites in methanol and ethanol fractions (Table-1). This is similar to the earlier reports. Asha *et.al*, extracted flavnoids and phenolics from the root extracts of *Acanthus ilicifolius* in ethanol.[20] The efficacy of antibacterial action was highest for methanol extracts in the stem extracts of *Acanthus ilicifolius*. In this regard the studied plant is very similar to other members of the mangroves. Govindasamy *et.al* who studied the phytochemical constituents of different mangroves in India summarized similar findings.[21] The presence of alkaloids, flavonoids and tannins in 3, 4 and 2 solvents respectively (Table-1) may be an important finding specially with respect to the scavenging of free radicals has been well studied.[22] Future studies should explore the potential applicability of the *Acanthus ilicifolius* extracts in the management of ageing and cancer. These are also known to be associated with other biological activities such as antibacterial, antifungal, antidiuretic activities.[23]

All the tested extracts of *Acanthus ilicifolius* stem possessed different levels of antibacterial activities against the tested strains. Methanol extracts had a broad spectrum antibacterial action. The zones of inhibitions for methanol extracts were higher for gram positive test cultures. The difference rate of inhibition activities appear to be directly related to the qualitative diversity of the compounds that are present in the extract. 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.[24] 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[25], 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 stem extracts of *Acanthus ilicifolius* in methanol and ethanol than that of the acetone and ethyl acetate in our study can be correlated with the medicinal preparation that use rum and liquour to extract the active plant components. Ethyl acetate extracts did not inhibit *Enterobacter cloacae, Klebsiella pneumonia, Staphylococcus aureus* and *Streptococcus pyogenes* however, negative results does not mean that bioactive principles are absent in the ethyl acetate extract. The bioactive principles may be insufficient to cross the membrane. Similar zone size of 13.66 mm was observed with acetone extract against *Enterobacter aerogenes, Escherichia coli and Klebsiella pneumonia*. This may indicate implication(s) of same/similar active compound(s) and/or same/similar mode of action(s) across these species. The inhibitory effect of gentamicin is superior to that of the tetracycline. This is similar to earlier reports of Odubanku[26] and Suganya.[27]

In our study, the MIC value for all the positive extracts against the tested bacteria were between 2.0mg/100µl to 7mg/100µl. Gram negative test cultures showed higher MIC values than Gram positive text cultures to the stem extracts in ethyl acetate and methanol. However, MIC values of stem extracts in ethanol are higher gram positive organisms than gram negative.

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.[28] 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[29] 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 ampicillin 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 antimicrobial 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*

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