

Research Article**Phytochemical screening, in vitro antioxidant activity, cytotoxicity study using Brine shrimp and antimicrobial study of *Acanthus ilicifolius* (Linn.) leaves**

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Abstract

Background: *Acanthus ilicifolius*, an associate mangrove also known as Holly mangrove is a shrub abundantly found in marshy areas and coastal areas that are frequently inundated with sea water. The leaves of this shrub are widely used by tribal people for medicinal purposes mainly treating snake bites and for curing rheumatism, asthma, diabetes, hepatitis, osteoporosis and inflammation. **Objective:** The study is intended to explore the phytochemical, antioxidant, cytotoxic and antimicrobial efficiency of *A. ilicifolius* leaves scientifically. **Materials and Methods:** In this study the methanol, hexane and chloroform extract of leaves of *A. ilicifolius* were subjected to qualitative screening of phytochemicals and quantitative estimation of their antioxidant capacity and cytotoxicity study using brine shrimp larvae along with antimicrobial efficiency by well diffusion method were done. **Results and Conclusion:** During the phytochemical screening the leaf extract was found to be rich in bioactive compounds. The LC50 value (for methanol 537.16 ± 8.3 µg/ml, hexane 691.84 ± 1.85 µg/ml and chloroform 776.27 ± 4.18 µg/ml) derived from probit regression method showed that *A. ilicifolius* leaf has low toxicity towards *Artemia nauplii*. The leaf extract showed good antioxidant activity during hydroxyl radical scavenging assay (IC50 for methanol 169 µg/ml, chloroform 2310 µg/ml and hexane 4336.6 µg/ml) and total antioxidant activity test (for hexane 15.31 ± 1.1 mg/g AAE, chloroform 20.24 ± 1.23 mg/g AAE and methanol 27.095 ± 2.17 mg/g AAE). Antimicrobial activity using well diffusion method showed that *A. ilicifolius* leaf extract is an effective antimicrobial agent with highest percentage inhibition against *Salmonella* (methanol and hexane extract) and *Mycobacterium* species (chloroform extract). The study establishes that *Acanthus ilicifolius* is potentially a good candidate for numerous medical applications.

Keywords: *Acanthus ilicifolius*, phytochemical, cytotoxicity, antioxidant, antimicrobial, *Mycobacterium*

Introduction

Mangroves for a long time have been the curiosity for scientists because of their unique biochemical properties and sought after by forest dwellers for various medical applications and fodder. *Acanthus ilicifolius*, an associate mangrove also known as Holly mangrove is a shrub abundantly found in marshy areas and coastal areas that are frequently inundated with sea water. This plant is widely used by tribal people for medicinal purposes mainly treating snake bites and for curing rheumatism, asthma, diabetes, hepatitis, osteoporosis and inflammation treatments.

Rich in bioactive compounds like phenolics, triterpenoids, flavonoids, lignin, terpenoids, steroids and alkaloids, studies have also reported *Acanthus ilicifolius* to prevent tumor growth and cancer progression (Firdaus, 2011; Bandarnayake, 1998; Govindswamy and Kannan, 2012; Sundaram et al., 2011; Kumar et al., 2008; Westmann and Liebezeit, 2008; Graham et al., 2000; Babu et al., 2002; Gamble, 2014; Udayan and Indira, 2009).

Materials and Methods**Collection of plant material**

The leaves of *Acanthus ilicifolius* were collected from Mangalavanam area (9°59'13" North and 76°16'26" East) in Ernakulam district during the month of May. The leaves were washed with distilled water to remove any debris and dust particles, shade dried for about 2 weeks and grounded in an electrical blender and stored in air tight bags in refrigerator (4°C).

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Authentication of *Acanthus ilicifolius*

The specimen was identified by the curator and voucher specimen was submitted in the Herbarium of Department of Botany, University of Kerala, Kariavattom with access number KUBH-6026.

Extraction using solvents

The dried leaves of *A. ilicifolius* were extracted using chloroform, hexane and methanol by Soxhlet extraction process (Veni, 2014; Amit and Shalini, 2014) followed by vacuum drying using rotavapour (Mayorga et al., 2010). The crude extracts were stored in refrigerator under air tight condition for further analysis.

Phytochemical screening

The presence of bioactive compounds was screened in leaf extract following the protocol of Harborne (Harborne, 1984).

Alkaloids

Dragendroff's test: To 0.5ml of alcoholic solution of extract added 2.0 ml of hydrochloric acid. To this acidic medium 1.0 ml of Dragendroff's reagent (solution of potassium bismuth iodide) was added. An orange red precipitate produced immediately indicates the presence of alkaloids.

Flavonoids

In a test tube containing 0.5 ml of alcoholic extract 5-10 drops of dilute hydrochloric acid and a small piece of zinc chloride ($ZnCl_2$) or magnesium (Mg) were added and the solution was boiled for few minutes. In the presence of flavonoids reddish pink or dirty brown color was produced.

Saponins

In a test tube containing 0.5 ml of aqueous extract, a drop of sodium bicarbonate was added. The mixture was shaken vigorously and kept for 3 minutes. A honey comb like froth was formed and it showed the presence of saponins.

Phenol

Ferric chloride test: To 2 ml of alcoholic solution of extract, 2 ml of distilled water followed by drops of 10% aqueous solution of ferric chloride ($FeCl_3$) solution were added. Formation of blue or green color indicates the presence of phenols.

Steroids

Salkowski test: To 2ml of extract, 2ml of chloroform and 2ml of concentrated sulphuric acid H_2SO_4 were added and shaken vigorously. Presence of red color indicated the presence of steroids.

Glycosides

A small amount of alcoholic extract was dissolved in 1 ml of H_2O and the aqueous NaOH solution was added. Formation of yellow

color indicates the presence of glycosides.

Tannins

Gelatin test: To 1-2 ml of extract 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicated the presence of tannins.

Terpenoids

To 2ml of extract added 2 ml of chloroform and 3ml of concentrated sulphuric acid was added carefully along the sides of the test tube. In the presence of terpenoids, reddish brown colour was produced.

Reducing sugars

Benedict's test: Equal volume of Benedict's reagent and test solution are mixed in a test tube and heated in boiling water bath for 5 minutes. Appearance of green, yellow or red indicated the presence of reducing sugar depending upon the amount.

Cytotoxicity assay using brine shrimp larvae

Artemia salina cysts purchased from CMFRI, Vizinjam were used for in vitro cytotoxicity assay. In vitro cytotoxicity test of methanol extract was performed on 24 hours old brine shrimp (*Artemia salina*) nauplii hatched in filtered sea water augmented with yeast powder and aerated throughout under the lamp maintaining the optimal conditions for nauplii (Mentor et al., 2014). 2.5 ml of different concentrations (50 μ g/ml, 100 μ g/ml, 200 μ g/ml, 400 μ g/ml, 800 μ g/ml and 1600 μ g/ml) of extract dissolved in 10% DMSO added in each well already containing 2.5ml sea water and 10 nauplii each. After 24 hours number of dead nauplii in each well was counted. Each concentration was tested in triplicate. The median lethal concentration (LC50) was obtained by plotting the percentage mortality of shrimp against logarithm of sample concentration (Meyer et al, 1982). LC50 values were derived from probit regression analysis using Finney's table (Finney, 1971). The toxicity of plant extract expressed as the LC50 values were valorized by comparison to Clarkson's toxicity index. Potassium dichromate was used as a positive control and DMSO was used as a negative control.

Antioxidant activity

Hydroxyl radical assay

This assay is based on the qualification of the degradation product of 2-deoxy ribose by condensation with thiobarbituric acid (TBA) (Halliwell et al., 1987; Elizabeth and Rao, 1990). Hydroxyl radical was generated by the Fe^{3+} - ascorbate- EDTA - H_2O_2 system (The Fenton reaction). Different concentration of extracts 125-2000 μ g/ml from a stock concentration of 10mg/ml mixed with 500 μ l reaction

mixture ((2-deoxy 2-ribose (2.8mM), FeCl₃ (100µm), EDTA (100µm), H₂O₂ (1.0mM), ascorbic acid (100µm) in KH₂PO₄ - KOH buffer (20 mM pH 7.4)) was made up to a final volume of 1 ml. A control without the test compound, but an equivalent amount of distilled water was taken. After incubation for 1 hour at 37°C, add 1ml of 2.8% TCA, then 1ml 1% aqueous TBA was added and the mixture was incubated at 90°C for 15 minutes to develop the color. After cooling the absorbance was measured at 532 nm against an appropriate blank solution. The IC₅₀ value was estimated after calculating percentage inhibition. Gallic acid was used as positive control.

$$\% \text{ inhibition} = \left\{ \frac{Ac-As}{Ac} \right\} \times 100$$

Ac – Absorbance of control

As – Absorbance of sample

Total antioxidant activity

Total antioxidant activity was evaluated by the phosphomolybdenum reduction assay method according to the protocol by Prieto, et al. (1999). The reduction of molybdenum, that is, Mo(IV) to Mo(V) by the leaf extract with subsequent formation of green phosphate/Mo(V) complex at acid pH. 1 ml of different extract concentration was mixed with 1 ml of reagent mixture (0.6 M Sulphuric acid, 28mM Sodium phosphate, and 4mM Ammonium molybdate) and incubated at 95°C for 90 minutes. The absorbance was measured at 695 nm using spectrophotometer. Ascorbic acid was used as positive control. Total antioxidant capacity was expressed as ascorbic acid equivalent (AAE).

Antimicrobial activity

Petriplates containing 20ml Muller Hinton Agar Medium were seeded with bacterial culture of *Acromonas hydrophilis*,

Escherichia coli, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus mutans*, *Mycobacterium fortuitum*, *Clostridium botulinum*, *Vibrio cholera* and *Bacillus cereus* (growth of culture adjusted according to McFards Standard, 0.5%). Potato dextrose agar was used as medium for fungal strains of *Aspergillus niger* and *Candida albicans*. Wells of approximately 10mm was bored using a well cutter and samples of 25 µl, 50µl, 100 µl concentrations were added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well (NCCLS, 1993). Streptomycin and clotrimazole was used as a positive control.

Statistical analysis

All the experiments were performed in triplicates. The values are represented in mean ± standard deviation. Comparison was done using t-test and regression analysis was done in MS Excel 2010.

Results and discussion

Phytochemical screening

The soxhlet extraction of *A. ilicifolius* yielded 9.1% of dried crude methanol extract also 0.15% and 2.64% for chloroform and hexane respectively. The phytochemical screening of the crude extract of *Acanthus ilicifolius* leaves indicated the presence of most of the bioactive compounds listed in table 1. Methanol is known to be most effective solvent for dissolving most of the bioactive compounds in plant materials and allows extraction of active compounds like anthocyanins, saponins, tannins, flavones and polyphenols (Babu et al., 2002; Tiwari et al., 2011). The studies done by Bandaranayake, 1998 and Poorna et al,

Table 1. Table showing the bioactive compounds and their indication of presence or absence in different extracts of *A. ilicifolius*.

Phytochemicals	Appearance of reactions	Inference		
		Methanol	Hexane	Chloroform
Alkaloids	Orange brown precipitate	+	-	-
Flavanoids	Yellow colored precipitate	+	+	-
Saponins	Honey comb like froth	++	++	++
Phenols	Bluish green color	++	-	-
Steroids	Reddish color of solution	++	++	++
Glycosides	Absence of Yellow color	-	-	+
Terpenoids	Reddish brown color	++	++	++
Tannins	Formation of white precipitate	+	++	-
Reducing sugars	Yellow color of solution	+	+	+

+ denotes presence of compound, - denotes absence and ++ denotes highly present

2011 revealed that they found *A. ilicifolius* as the rich source of long chain alcohols, terpenes, steroids and triterpenoidal saponins.

Cytotoxicity assay

Among the three different extracts chloroform extract showed highest LC50 value and methanol showed lowest LC50 value, the values were compared at the confidence interval of 95%. Yet, all the three extracts showed to be lying in the low toxicity range according to Clarkson's toxicity index (Clarkson et al., 2004). The LC50 of potassium dichromate which was used as positive control was found to be 15.5 ± 1.2 $\mu\text{g/ml}$ and that of negative control in which sea water only was used, no mortality was found. Based on Clarkson's toxicity index, the LC50 value of leaf extract belong to low toxicity range (Clarkson et al., 2004). Table 2 lists LC50 value for each crude extract and positive control.

Antioxidant activity

The sample exhibited hydroxyl radical scavenging in a dose dependent manner in the range of 125-2000 $\mu\text{g/ml}$ in the reaction mixture. The *A. ilicifolius* leaf extract showed radical scavenging activity in the order Methanol > Hexane > Chloroform (Figure 1).

The total antioxidant potential of sample was investigated and compared against standard Ascorbic acid ($R^2 = 0.9808$). Table 3 shows the ascorbic acid equivalent of each extract and their IC50

Table 2. Table showing the concentration of extract required to bring the mortality of Brine shrimp larvae to 50%.

Solvent extract	LC50 ($\mu\text{g/ml}$)
Hexane extract	691.84 \pm 1.85
Methanol extract	537.16 \pm 8.3
Chloroform extract	776.27 \pm 4.8
Potassium dichromate	15.5 \pm 1.2

Values are represented as mean standard deviation. Different superscripts indicate significantly different values ($p < 0.05$).

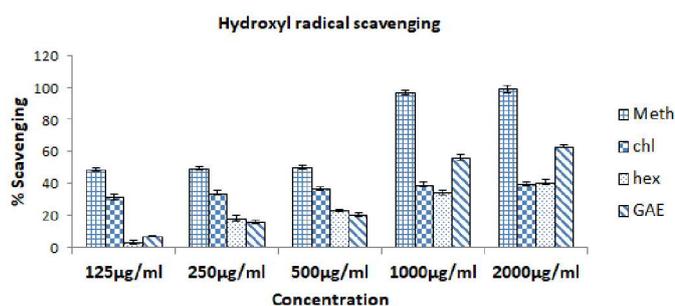


Figure 1. Graph shows percentage scavenging of each extract at different concentrations and gallic acid is used as standard

Table 3. Table showing ascorbic acid equivalent activity and concentration at 50% hydroxyl radical scavenging activity of each extract.

Solvent extracts	AAE ($\mu\text{g/ml}$)	50% hydroxyl radical scavenging activity ($\mu\text{g/ml}$)
Methanol	27.095 \pm 2.17	169
Chloroform	20.242 \pm 1.23	4336.6
Hexane	15.31 \pm 1.1	2310

values. Also, figure 2 shows the increasing trend of absorbance with increase in concentration for each extract. The result shows that methanol has 27.095mg/g of Ascorbic acid (Vitamin C).

Antimicrobial activity

Inhibition zones were found around agar wells at varying sizes for all the three extracts. Table 4 shows the percentage inhibition against positive control for methanol, chloroform and hexane extracts towards each microorganism.

Methanol extract was found to be effective against *Salmonella typhimurium* and *Mycobacterium fortuitum* then *Klebsiella pneumonia* and least effective against *Vibrio cholera* and *Staphylococcus aureus*. Chloroform extract was effective against *Mycobacterium fortuitum* and least effective against *Vibrio cholera* and *Staphylococcus aureus*. Hexane extract was found to be equally active

Table 4. Table showing percentage inhibition of methanol, chloroform and hexane extract against micro-organisms.

Microorganisms	Percentage inhibition		
	ME	CE	HE
<i>Aeromonas hydrophilis</i>	37.83	33.3	34.8
<i>Escherichia coli</i>	51.5	29.95	38.3
<i>Klebsiella pneumonia</i>	73.35	38.55	52.3
<i>Pseudomonas aeruginosa</i>	61.65	51.65	58.3
<i>Staphylococcus aureus</i>	28.6	28.6	34.95
<i>Streptococcus mutans</i>	30.6	32.77	39.05
<i>Mycobacterium fortuitum</i>	82.5	90	90
<i>Clostridium botulinum</i>	54	64	54
<i>Vibrio cholera</i>	28.75	27.5	30
<i>Bacillus cereus</i>	60	85	52.5
<i>Salmonella typhimurium</i>	87.5	70	92.5
<i>Aspergillus niger</i>	48.05	46.05	59.55
<i>Candida albicans</i>	51.735	46.63	46.51

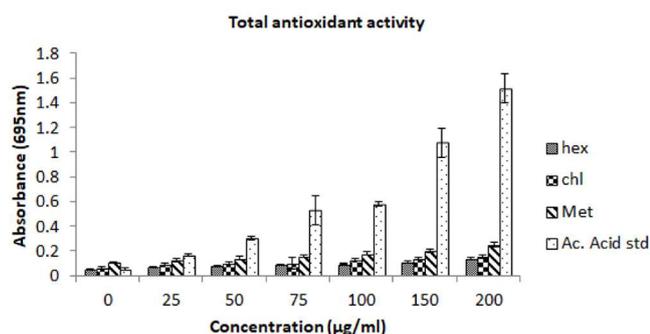


Figure 2. Graph shows increasing trend of absorbance with increasing concentration of different extracts and ascorbic acid used as standard.

against *Mycobacterium fortuitum* and *Salmonella typhimurium* and least effective against *Aeromonas hydrophila*. In a study conducted by Chinnavenkataraman and Mani, 2013, it is reported that chloroform extract of *A. ilicifolius* showed maximum activity against *Staphylococcus*, *Streptococcus*, *Pseudomonas* and *Candida* strains meanwhile methanol showed maximum activity against *Staphylococcus* strains. Study done by Poorna et al, 2011 also proved that *A. ilicifolius* plant is active against *K. pneumoniae*, *E. coli* and *V. cholera* also methanol to be effective against *S. typhi* and *C. albicans*. Therefore it is clear that the *A. ilicifolius* leaves have good inhibitory effect on pathogenic microorganisms.

Conclusion

Acanthus ilicifolius leaves are reservoir of potential bioactive compounds with low in-vitro toxicity towards brine shrimp. In this study methanol extract showed significant radical scavenging activity and total antioxidant activity compared to other two extracts and therefore it can be concluded that the leaf has good antioxidant capacity and many studies also substantiates its exploitation in numerous medical applications. *A. ilicifolius* extracts were found to be effective against pathogens like mycobacterium, clostridium, salmonella etc. rendering it effective antimicrobial agent. Hence the *Acanthus ilicifolius* leaf proves to be an efficient candidate for further studies in pharmacology and cancer studies.

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Conflict of interest

There are no conflicts of interest.

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