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Research Article

Quantitative Phytochemical Analysis, *In vitro* Reducing Power and Anti-oxidant Activity of Methanol Leaf Extract of *Acanthus ilicifolius*

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ABSTRACT

Objective: The objective of this study was to estimate the phytochemical constituents of the methanol leaf extract of *Acanthus ilicifolius* and to examine reducing power with antioxidant activity of the extract by *in vitro* method.

Methods: Leaf extract was prepared by maceration method. Then the qualitative and quantitative phytochemical analysis of these extracts was carried out by applying standard procedures. Reducing power of the prepared extract was determined by Oyaizu method. In order to determine the *in vitro* scavenging ability of the extract against free radicals, anti-oxidant activity was determined by DPPH method.

Results: The extract was found to be rich in phytochemicals. The content of alkaloids and saponins was 16.26 ± 0.22%, 3.82 ± 0.06% respectively. The total phenolics (292.62 ± 0.50mg/g of GAE) and flavonoids (44.15 ± 0.30 mg/g of QE) was found to be higher in this extract. It showed that the plant extract had a higher level of antioxidant and reducing property even at lower concentrations due to its hydrogen donating capability.

Conclusion: The present study proves that the methanol leaf extract of *Acanthus ilicifolius* is a potent antioxidant and having high reducing power. Hence this extract found to be a valuable source of drugs to treat diseases caused by oxidation.

Keywords: Phytochemical, Methanol extract, Maceration, Phenolics, flavonoids, DPPH, Antioxidant, Reducing power assay.

INTRODUCTION

Mangrove plants are commercially used as fuel and timber in coastal areas for a long period of time and are lesser known for therapeutic usage[1]. They are rich source of medicines and produce wide range of phytochemicals because of their ecosystem which consist of heterogeneous habitats[2]. Based on World Health Organization data, more than 80% of world inhabitants depend on plant for their medicine and mangroves have been widely used for that purpose[3,4]..*Acanthus ilicifolius* is an ever green important medicinal plant from mangroves but its intact medicinal value has not been fully explored as yet. *Acanthus ilicifolius* belongs to Acanthaceae family and is known as kayzimulli, attumulli and kazhuthai mulli in vernacular. It is widely distributed in India, Malaysia and South Asian countries and used in traditional Indian and Chinese medicinal system to treat dyspepsia, paralysis, and skin disease and wound healing[5]. Various parts of this plant were scientifically explored for their biological activities like hepatoprotective[6], anti osteoporotic activity[7], antimicrobial[8], anticancer[9], analgesic[10], anti inflammatory[11], antidiabetic[12], antiulcer[13], antinociceptive[14] and lesmanicidal activity[15].

According to literature survey most of the adverse reactions and diseases are associated with oxidative stress which is produced by free radicals. Free radicals are generated due to normal metabolic and physiological reactions. These free radicals are highly reactive which can damage normal cells and that leads to a variety of diseases. Free radicals can be deactivated and neutralized by the novel compounds known as antioxidants[16]. Medicinal plants are rich source of antioxidants and the search for natural, effective and non-toxic antioxidants with lesser side effects is a still challenge for researchers. The search for a new therapeutic agent with antioxidant and reducing power is an interesting part of research. Hence there is a great attention towards antioxidants from plants which is rapidly increased in recent years. The aim of this study was to quantify the phytochemicals with antioxidant and reducing power activity present in methanol leaf extract of *Acanthus ilicifolius* by *in vitro* studies.

MATERIALS AND METHODS

Chemicals and reagents

2, 2-Diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, standards ascorbic acid, Gallic acid, and quercetin were purchased from Himedia Laboratories, Mumbai. All other chemicals and reagents were used in the present study of AR grade and purchased locally.

Collection of plant materials

The leaves of *Acanthus ilicifolius* were collected freshly from Parangippettai area, Cuddalore district, Tamilnadu during the month of September 2014 and the plant specimens were authenticated by Dr. Kathiresan, Professor

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& Director, CAS Marine Biology, Annamali University, Chidambaram, Tamilnadu.

Preparation of crude extract

The fresh leaves were washed thoroughly, cut into small pieces, dried in shade and ground well in mechanical grinder; then passed through the mesh to get uniformly coarse powder. This fine powder was stored in an air tight container. The methanolic leaf extract was prepared from 100g powdered plant material with 400 ml of methanol and kept for maceration for 72 hrs at room temperature. The extract was filtered and concentrated by rotary vacuum evaporator at 45˚C. The percentage yield of extract was calculated from the weight of final residue obtained.

Preparation of standard solutions

10mg of ascorbic acid, Gallic acid and quercetin were dissolved in exactly 10 ml of methanol and used as stock solution. From the stock solution, different concentrations were prepared in methanol and used to calibrate standard curve.

Table 1: Effect of methanolic leaf extract of *Acanthus ilicifolius* on the inhibition of DPPH free radical as compared to standard ascorbic acid

Conc (µg/ml)	% Inhibition of DPPH free radical	
	Ascorbic acid (standard)	Methanolic leaf extract of *Acanthus ilicifolius*
20	31.77±0.19	23.47±0.65*
40	44.82±0.34	32.06±0.43*
60	54.30±0.21	43.60±0.32*
80	63.54±0.64	56.45±0.16*
100	76.25±0.33	68.65±0.50*

Values are expressed as Mean ± SEM (n = 3) and p value is ≤ 0.01*Values are significant with p – value ≤ 0.01

Qualitative phytochemical analysis

Qualitative phytochemical analysis of methanolic leaves extract of *Acanthus ilicifolius* was carried out to identify the secondary metabolites by following the standard procedures[17, 18,19].

Quantitative phytochemical analysis

Alkaloides, phenolics, flavonoides and saponins were quantified by the following methods.

Determination of Alkaloids

5 gram of extract was dispersed in 50 ml of 10% acetic acid solution in methanol and was allowed to stand for 4 hrs at 28˚C. The resulting mixture was filtered through Whatmann filter paper. The obtained filtrate was evaporated in a boiling water bath to get a concentrated filtrate. The filtrate was treated with concentrated ammonium hydroxide drop by drop until the precipitation of the alkaloids is over. The precipitate was filtered through a weighed Whatmann filter paper. Then the residue was thoroughly washed with 1% ammonia and kept in hot air oven at 70˚C. The percentage of alkaloid was calculated from the dry weight of the residue[20].

Determination of saponins

10g of plant extract was dispersed in 100 ml of 20% ethanol and the mixture was kept in a water bath for 4 hours with continuous stirring at about 55ºC. It is filtered and the resulted filtrate was mixed with 100 ml of 20% ethanol and kept in a boiling water bath to reduce the volume. The concentrated filtrate was transferred to a separating funnel and 100 ml of diethyl ether was added and shaken vigorously to purify the aqueous layer. The aqueous layer was treated twice with 50 ml of 5% aqueous sodium chloride and heated in a water bath. The residue was dried in an oven and the percentage of saponin was calculated[21].

Determination of total phenolics

Different concentrations (20, 40, 60, 80,100 µg/ml) of Gallic acid standard and two concentrations 50mg/ml and 100 mg/ml of plant extract were prepared; 1 ml of each sample and standard were placed in different test tubes. Then 2.5 ml of a 10% Folin-Ciocalteu reagent and 2 ml of 2% sodium carbonate were added and the test tubes were covered with aluminum foil. This mixture was incubated for 30 minutes at room temperature and the absorbance of standard gallic acid and the plant extract were measured spectrophotometrically at 765 nm. The total phenolic content of plant sample was calculated from the Gallic acid standard curve. The results were expressed as mg/gram of Gallic acid equivalents[22].

Determination of the total Flavonoids

The content of total flavonoids was done by aluminum chloride method. In this method the calibration curve was drawn from different concentrations of standard quercetin 2 to 10µg/ml and methanol leaf extract of *Acanthus ilicifolius* were prepared. 1 ml of each sample was mixed with 4 ml of distilled water in a volumetric flask and 300 µl of 5% sodium nitrate was added and incubated for 5 minutes at room temperature. Then 300 µl of 10% aluminum chloride was added to flask and 2 ml of 1 % sodium hydroxide was added immediately. The volume is then made up to 10 ml with distilled water. The absorbance was taken at 510 nm using UV-visible spectrophotometer. The results are expressed as mg/g of quercetin equivalent[23].

Determination of total antioxidant activity by in vitro method

The free radical scavenging capacity of the methanol leaf extract of *Acanthus ilicifolius* was determined by DPPH method. In this method ascorbic acid was used as a standard. Different concentrations (20, 40, 60, 80,100 µg/ml) of ascorbic acid and methanol extract were prepared. 0.5ml of 0.3 mM DPPH in solution was mixed with 100 µl of methanol extract and ascorbic acid. This mixture was kept at dark for 30 minutes at room temperature. The absorbance was measured at 517 nm. The free radical scavenging ability of methanol extract was calculated by using following formula[24],

$$\% \text{ inhibition} = \frac{\text{Abs. control} - \text{Abs. of sample}}{\text{Abs. of control}} \times 100$$

All the tests were done in triplicates.

In vitro reducing power assay

The reducing power of methanolic leaves extract of *Acanthus ilicifolius* was determined by Oyaizu method. Different concentrations (50 to 250µg/ml) of ascorbic acid and methanolic leaf extract were prepared. Each test sample was mixed with 2.5 ml of 0.2M phosphate buffer (pH 7.4) and 2.5 ml of 1% potassium ferricyanide and the mixture was incubated for 20 minutes at 55˚C. 2.5 ml of trichloro acetic acid was added and centrifuged at 3000 rpm for 10 minutes. Then 2.5 ml of upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride solution. The absorbance was measured at 700 nm[25].

Statistical analysis

MS office Excel 2007 was used to plot standard curve for TPC and TFC and to calculate linear regression equation and linear correlation coefficient (R^2) for a standard curve of Gallic acid and quercetin. All the experiments were performed in triplicates and the values are represented as Mean ± Standard Error Mean (SEM). The results were found to be significant with P value of ≤ 0.05 and P* is ≤ 0.01

RESULTS

Qualitative phytochemical analysis

The qualitative phytochemical analysis carried out on the leaves from the methanol extract of *Acanthus ilicifolius* revealed the presence of medicinally important constituents such as alkaloids, saponins, phenolics, flavonoids, steroids, cardiac glycosides, tannins, triterpenoids, anthroquinons, and the absence of resins and gums.

In this study, the phytochemical constituents were quantitatively determined for the content of alkaloids, saponins, total phenolics and flavonoids. The alkaloid content of the extract was 16.26 ± 0.22% and the content of the saponins was 3.82 ± 0.06%. This shows highly possible medicinal values of the extract.

Total phenolic content (TPC)

The amount of total phenolics was quantitatively determined by using Gallic acid as standard compound and

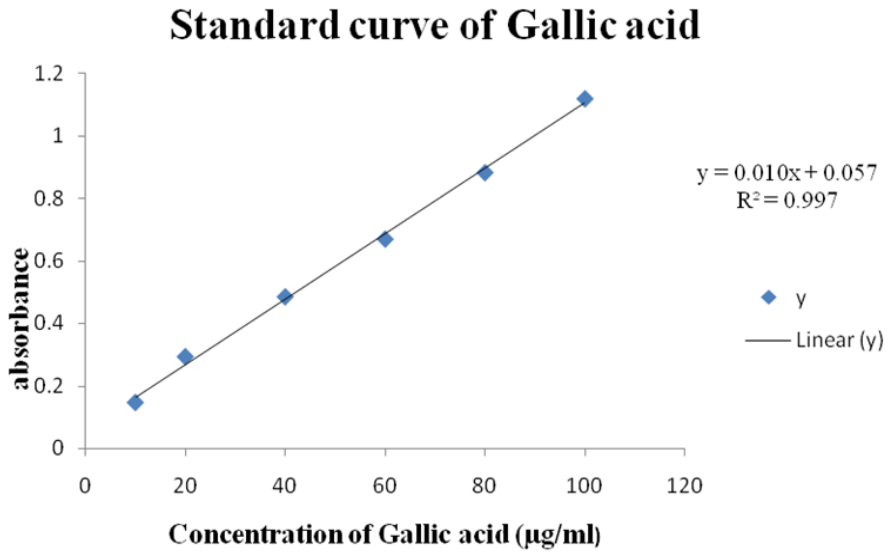

Figure 1: Calibration curve of Gallic acid. Each point represents the mean of three experiments

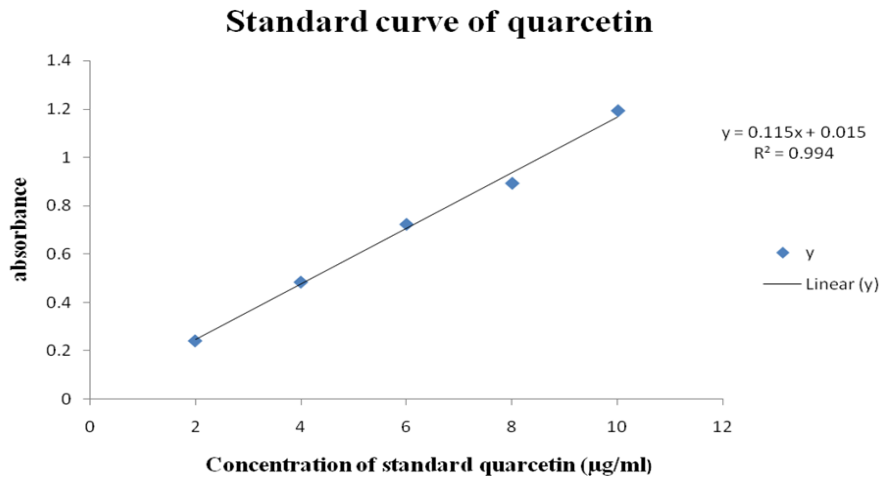

Figure 2: Standard curve of quercetin. Each point represents the mean of three experiments

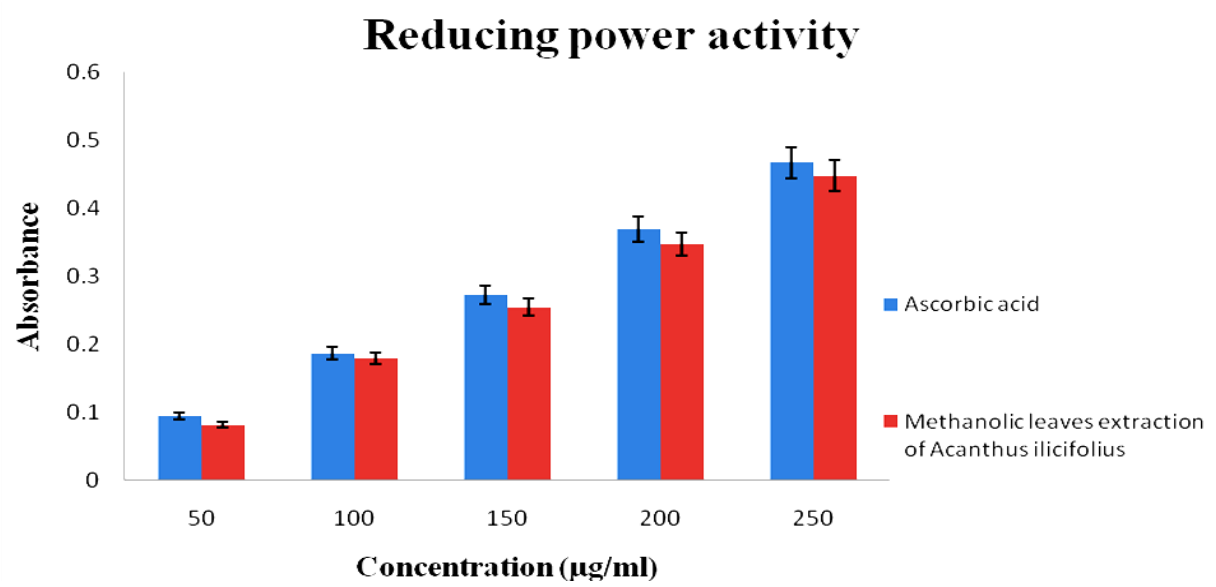

Fig. 3: Reducing power activity of methanolic leaf extract of *Acanthus ilicifolius* as compared to standard ascorbic acid. The values are the mean for a set of three values. The SEM is indicated by vertical bars.

the total phenol was expressed as mg/ gram Gallic acid equivalent(GAE) using the standard curve equation (i.e.) y = 0.010x + 0.057 and the linear correlation co- efficient (R^2) = 0.997. Y is the absorbance of Gallic acid extract at 760 nm. X is the TPC present in the extract, that can be calculated from the slope (m) = 0.010 and the intercept(c) = 0.057. The TPC of the methanol leaves extract was 292.62 ± 0.50mg/g of GAE.

Total flavonoids content (TFC)

Quercetin at different concentrations was used to calibrate the standard curve for the presence of TFC in methanolic leaf extract of *Acanthus ilicifolius* and the value is expressed as mg/g of quercetin equivalents (QE). The equation used was y = 0.115x+0.015 and the R^2 = 0.994. From this equation, the unknown flavonoid content in the extract was calculated and the TFC value was 44.15 ± 0.30 mg/g of QE.

Antioxidant activity

The results of DPPH assay are summarized in table 1. The results are expressed in percentage of inhibition of DPPH free radical and compared with standard ascorbic acid. The results describe that the antioxidant activity increases with increase in concentration. On comparison with ascorbic acid, plant extracts exhibited a high antioxidant activity (68.65 ± 0.50% at 100µg/ml).

Reducing power assay

The reduction of the Fe^{3+} (ferric cyanide complex) to Fe^{2+} (ferrous cyanide) by the antioxidants of methanolic leaf extract of *Acanthus ilicifolius* was carried out. Depending upon the reducing ability, the antioxidant color changes from pale green to blue and was measured at 700nm. The results were compared with standard ascorbic acid and depicted in figure 3. Methanol leaf extract of *Acanthus ilicifolius* showed very low reducing power when compared with standard.

DISCUSSION

Presence of phytochemicals, antioxidant activity, reducing power activity and other biological properties are highly dependent on solvent medium used. Methanol is more effective solvent which can easily penetrate the cellular membrane and dissolves intracellular components present in plant cells. According to this, the results of qualitative analysis show that methanol leaf extract of *Acanthus ilicifolius* is rich in phytochemicals. In general, the alkaloids have biological activities such as antimalarial, antimicrobial, antihyperglycemic, anti-inflammatory and pharmacological effect[26,27]. The saponins present in the plant extract exhibit various pharmacological functions including maintenance of cell membrane permeability[28], anti-cholesterolic[29], stimulation of luteinizing hormone release, anticancer effect[30], anti-hyperglycemic and adjuvant for vaccines[31]. The alkaloids and saponins are present in considerable amount in the leaf extract of *Acanthus ilicifolius* (16.26 ± 0.22%, 3.82 ± 0.06%) respectively. Hence they can be considered for usage in therapeutics.

Free radicals are triggers for oxidative stress and cause diseases like arthritis, cancer, diabetes etc[32]. Oxidative Stress is created due to the imbalance between free radicals and endogenous antioxidants that can be rectified by the intake of exogenous antioxidants[33]. Antioxidant compounds from natural source can interfere with free radicals and break the reaction chain which can prevent the cellular damage. The current study shows the presence of considerable amount of TPC (292.62 ± 0.50mg/g of GAE) and TFC (44.15 ± 0.30 mg/g of QE). The phenolics and flavonoids of plants directly influence the antioxidant property due to their hydroxyl groups which has scavenging ability for free radicals[34]. The methanolic leaf extract shows highest antioxidant activity with the percentage of 68.65±0.50% against DPPH radicals, which

indicates the presence of hydroxyl group and their scavenging ability. The extract shows the higher absorption when compared with standard ascorbic acid. The high absorbance indicates the high reducing power. The present experiment reveals a direct correlation between reducing power and antioxidant activity of the extract.

Antidiabetic activity of methanolic leaf extract of *Acanthus ilicifolius* has been proved by *in vitro* method. This study also exhibited antioxidant and reducing power activity even at the lower concentrations of methanolic leaf extract of *Acanthus ilicifolius*.

CONCLUSIONS

The present study suggests that the methanol leaf extract of *Acanthus ilicifolius* are rich in Phytochemicals which accounts for antioxidant and reducing power activity. Many reports explain that most of the antioxidant possess anti inflammatory, antiviral, antidiabetic and antimicrobial activity. Hence this extract may have the possibility to use as drug with antioxidant property to treat diseases related to oxidative stress. Further studies for the isolation, purification and characterization of phytochemicals are in progress.

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CONFLICT OF INTEREST

The authors wish to declare that they have no conflict of interest.

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