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Antibacterial, Cytotoxic and Antioxidant Activity of Crude Extract of *Marsilea Quadrifolia*

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Abstract

The main aim of this study was to find out the antibacterial, antioxidant and cytotoxic activity of petroleum ether, chloroform and ethyl acetate extracts of *Marsilea quadrifolia* (Family: Marsileaceae). For antibacterial test, Disc diffusion technique was used against 5 Gram positive and 11 Gram negative human pathogenic bacteria. The range of zone of inhibition of chloroform and ethyl acetate extracts was 9 to 20 mm. The petroleum ether extract did not show any zone of inhibition against any tested pathogenic bacteria. The Brine shrimp lethality bioassay method was used to determine the cytotoxicity activities and Vincristin sulphate was used as a positive control. The LC_{50} values of standard Vincristin sulphate, petroleum ether, chloroform and ethyl acetate extracts were 6.628µg/ml, 9.543µg/ml 7.820 µg/ml, and 8.589µg/ml respectively. All the fractions showed potent antioxidant activity, of which the ethyl acetate fraction demonstrated the strongest antioxidant activity with the IC_{50} value of 50.1053 µg/ml.

Keywords: *Marsilea quadrifolia*, Marsileaceae, antioxidant,

1. Introduction

Many higher plants accumulate extractable organic approaches substances in quantities sufficient to be economically management of disease. 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. It is estimated that only one percent of 2, 65,000 flowering plants on earth have been studied exhaustively for their chemical composition and potential against important medicinal value (Cox et al., 1994). In many developing countries, traditional medicine is one of the primary health care systems (Fransworth, 1993; Houghton, 1995). Herbs are widely exploited in the traditional medicine and their curative potentials are well documented (Dubey, et al., 2004).Large scale evaluation of the local flora exploited in traditional medicine for various biological activities is a necessary first step in the isolation and characterization of the active principle and further leading to drug development. In view of these *M. quadrifolia* plant was studied exhaustively for its potential against important sixteen human pathogenic bacteria, antioxidant and cytotoxic effects. *M. quadrifolia* is an aquatic fern bearing 4 parted leaf resembling '4-leaf clover' (Trifolium). Leaves floating in deep water or erect in shallow water or on land. Leaflets obdeltoid, to 3/4" long, glaucous, petioles to 8" long; Sporocarp (ferns) ellipsoid, to 3/16" long, dark brown, on stalks to 3/4" long, attached to base of petioles. A juice made from the leaves is diuretic and febrifuge and also used to treat snakebite and applied to abscesses etc (Duke et al., 1985). The plant is anti-inflammatory, diuretic, depurative, febrifuge and refrigerant (Duke et al., 1985; Schofield, 1989). The plant contains an enzyme named Thiaminase (Schofield, 1989).

2. Materials and Methods

2.1. Plant Material

The plant, *M. quadrifolia* was collected from Rajshahil in the month of March 2009 and identified by Dr. M.A. Razzaque Shah, Tissue Culture Specialist, BRAC Plant Biotechnology Laboratory, Bangladesh.

2.2. Plant Material Extraction

The aerial parts of plant were collected, sun dried for seven days and ground. The dried powder of *M. quadrifolia* (200gm) was soaked in 600ml of ethanol for 7 days in cold condition and filtered through a cotton plug followed by Whatman filter paper number 1 followed by solvent-solvent partitioning with petroleum ether, chloroform and ethyl acetate (Haque et al., 2008).

2.3. Antibacterial assay

The disc diffusion method (Bauer et al., 1966) was used to test antimicrobial activity against sixteen bacteria (table-1). Solutions of known concentration (mg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amounts of the test substances using micropipette. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs (Kanamycin 30µg/disc) and blank discs (impregnated with solvents) were used as a positive and negative control. These plates were then kept at low temperature (4ºC) for 24 h to allow maximum diffusion. There was a gradual change in concentration in the media surrounding discs. The plates were then incubated at 37ºC for 24 h to allow maximum growth of the organisms. The test materials having antibacterial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the medium. The antibacterial activity of the test agent was determined by measuring the diameter of zone of

inhibition expressed in millimeter. The experiment was carried out three times and the mean of the reading is required (Bauer et al., 1966).

2.4 Cytotoxicity Screening

Brine shrimp lethality bioassay is widely used in the bioassay for the bioactive compounds (Meyer et al., 1982; Zhao et al., 1992). Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. The eggs of the brine shrimp were collected from an aquarium shop (Dhaka, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) for 48 hr to mature shrimp called nauplii. The cytotoxicity assay was performed on brine shrimp nauplii using Meyer method (Meyer, et al., 1982).The test samples (extract) were prepared by dissolving them in DMSO (not more than 50 µl in 5 ml solution) plus sea water (3.8% NaCl in water) to attain concentrations of 5µg/ml, 10µg/ml, 20µg/ml, 40µg/ml, and 80µg/ml. A vial containing 50µl DMSO diluted to 5ml was used as a control. Standard Vincristine sulphate was used as positive control. Then matured shrimps were applied to each of all experimental vials and control vial. After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial were counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

2.5. Screening for antioxidant activity

Antioxidant activities of the aerial part of methanol extract was determined on the basis of their scavenging potential of the stable DPPH free radical in both qualitative and quantitative assay.

i) **Qualitative assay:** A suitably diluted stock solutions were spotted on pre-coated silica gel TLC plates and the plates were developed in solvent systems of different polarities (polar, medium polar and non-polar) to resolve polar and non-polar components of the extracts. The plates were dried at room temperature and were sprayed with 0.02% DPPH in ethanol. Bleaching of DPPH by the resolved band was observed for 10 minutes and the color changes (yellow on purple background) were noted (Sadhu et al., 2003).

ii) **Quantitative assay**: The antioxidant activity of the aerial part extract of *M. quadrifolia was* determined using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay by the method of Blois (1958). DPPH offers a convenient and accurate method for titrating the oxidizable groups of natural or synthetic anti-oxidants (Cao et al., 1997). DPPH solution was prepared in 95% methanol. The crude extracts of *M. quadrifolia were* mixed with 95% methanol to prepare the stock solution (5 mg/50mL). The concentration of the sample solutions was 100µg/ml. The test samples were prepared from stock solution by dilution with methanol to attain a concentration of 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml & 100µg/ml respectively. Freshly prepared DPPH solution was added in each of these test tubes containing *M. quadrifolia* extract and after 20 min, the absorbance was taken at 517 nm. Ascorbic acid was used as a positive control. The DPPH solution without sample solution was used as control. 95% methanol was used as blank. Percent scavenging of the DPPH free radical was measured using the following equation-

% DPPH radical scavenging (%) = [1-(As/Ac)] ×100.

Here, Ac=absorbance of control, As =absorbance of sample solution.

Then % inhibitions were plotted against respective concentrations used and from the graph IC_{50} was calculated.

3. Results

3.1. The Results of Antibacterial Screening

The petroleum ether, chloroform and ethyl acetate crude extracts (500 µg/disc) of the aerial part of *M. quadrifolia* were screened against sixteen human pathogenic bacteria to check antibacterial activities

by disc diffusion method. The petroleum ether extract (500µg/disc) of *M. quadrifolia* showed no activity against the tested pathogenic organisms. On the other hand chloroform crude extracts (500µg/disc) showed excellent antibacterial activity with the average zone of inhibition of 11-20 mm by disc diffusion method (table 1), among the tested bacteria, the growth of *Pseudomonas aeruginosa* (20 mm) was highly inhibited. In case of ethyl acetate extract the highest activity was also seen against the growth of *P. aeruginosa* having the zone of inhibition of 18mm. Besides this, the chloroform and ethyl acetate crude extracts showed good activity against the growth of *Staphylococcus aureus* (18mm), *S. dysenteriae* (16mm) *S. shiga* (14mm) and *S. boydii* (14 mm).

Table 1: *In vitro* antibacterial activity of *M. quadrifolia* and standard Kanamycin discs

Test organisms	Diameter of zone of inhibition			
	Petroleum ether extract (500µg/disc)	**Chloroform extract (500µg/disc)**	**Ethyl acetate extract (500µg/disc)**	**Kanamycin (30µg/disc)**
Gram positive bacteria				
Bacillus megaterium	-	16	13	30
Bacillus subtilis	-	16	15	23
Bacillus cereus	-	15	14	22
Staphylococcus aureus	-	18	11	26
Sarcina lutea	-	11	9	24
Gram negative bacteria				
Escherichia coli	-	11	10	22
Pseudomonas aeruginosa	-	20	18	25
Salmonella paratyphi	-	14	10	25
Salmonella typhi	-	16	11	25
Shigella boydii	-	12	14	25
Shigella sonnei	-	11	12	24
Shigella shiga	-	13	14	23
Shigella flexneri	-	13	12	22
Shigella dysenteriae	-	16	16	25
Vibrio mimicus	-	11	7	28
Vibrio parahaemolyticus	-	11	7	26

Note: "-" sign indicates no activity.

3.2. The Results of Brine Shrimp Lethality Bioassay

Following the procedure of Meyer, the lethality of the crude petroleum ether, chloroform and ethyl acetate extracts of *M. quadrifolia* to brine shrimp was determined on *A. salina* after 24 hours of exposure the samples and the positive control, vincristine sulphate. This technique was applied for the determination of general toxic property of the plant extractive. The LC_{50} values for standard vincristine sulphate and extracts of *M. quadrifolia* were presented in table 2. The chloroform extract of plant showed the lowest LC_{50} value and petroleum ether extract showed highest value which was 7.820µg/ml and 9.543 µg/ml respectively (Fig-1).

Figure 1: Determination of LC_{50} values for standard crudepetroleum ether, chloroform and ethyl acetate extract of *M. quadrifolia* from linear correlation between logarithms of concentration versus percentage of mortality.

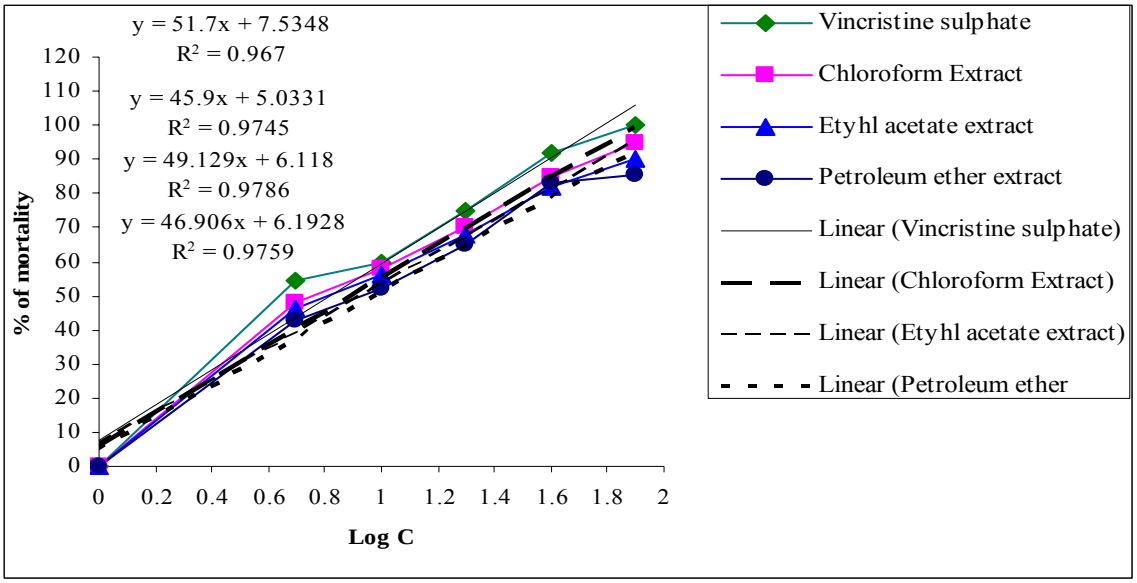

Table 2: LC_{50} data of test samples of *M. quadrifolia* and Vincristine sulphate

Samples	LC_{50} (µg/ml)
Vincristine sulphate	6.628
Petroleum ether extract	9.543
Chloroform extract	7.820
Ethyl acetate extract	8.589

3.3. The Result of Antioxidant Activity

DPPH is one of the free radicals widely used for testing preliminary radical scavenging activity of a compound or a plant extract.

i) **Qualitative assay**: The color changes (yellow on purple background) on the TLC plates were observed due to the bleaching of DPPH by the resolved bands.

ii) **Quantitative assay**: All the three extracts exhibited potential antioxidant activity. The petroleum ether extract of *M. quadrifolia* scavenged 50% DPPH free radical at the lowest inhibitory concentration (IC_{50}: 46.9312 9µg/ml). The chloroform, ethyl acetate extracts of the plant also revealed strong antioxidant activity IC_{50}: 49.4049 µg/ml and 50.1053 µg/ml respectively. These results denote the presence of antioxidant principles in the extractives.

Figure 2: Determination of LC_{50} values for standard and crude chloroform extract of *M. quadrifolia* from linear correlation between concentrations (µg/ml) versus percentage of scavenging of DPPH.

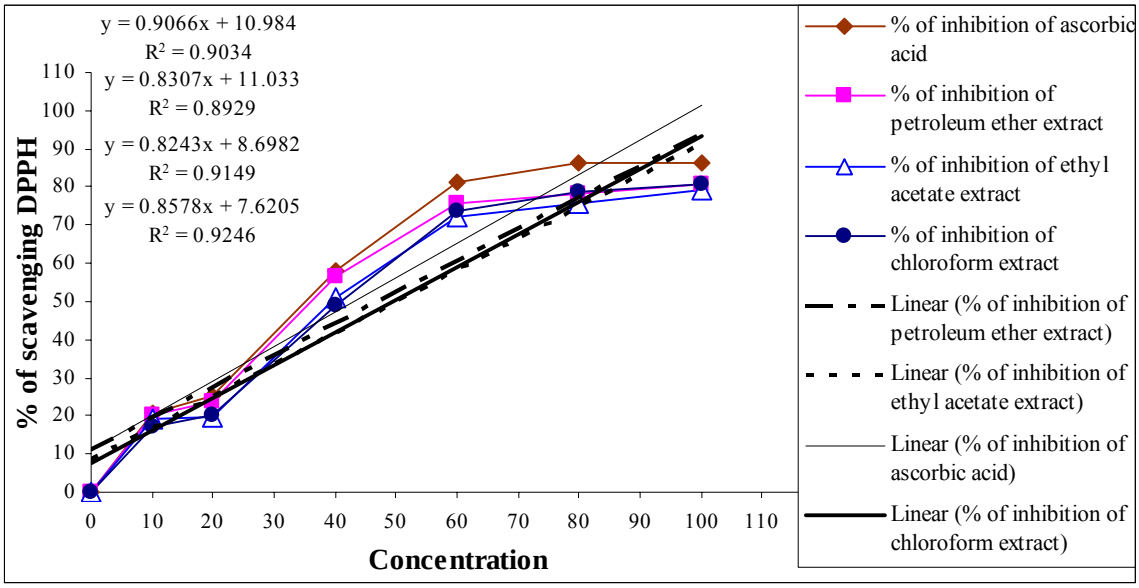

Table 3: IC_{50} data of test samples of *M. quadrifolia* and Ascorbic acid

Samples	**IC_{50} (µg/ml)**
Ascorbic acid	43.0356
Petroleum ether extract	46.9312
Chloroform extract	49.4049
Ethyl acetate extract	50.1053

4. Summary and Concluding Remarks

The present study indicated that the chloroform and ethyl acetate extracts of the aerial part of *M. quadrifolia have* got profound antibacterial, cytotoxic and antioxidant effect and may have potential use in medicine.

In comparison with the positive control (vincristine sulphate), the cytotoxicity exhibited by the petroleum ether, chloroform and ethyl acetate crude extracts of the plant showed potent activity. This clearly indicates the presence of potent bioactive principles in these crude extracts which might be very useful as antiproliferative, antitumor, pesticidal and other bioactive agents (Meyer et al., 1982). The free radical scavenging property may be one of the mechanisms by which this plant is effective in its ethno pharmacological uses against different ailments. Further studies comprising of phytochemical investigations of the used plant and evaluation for antioxidant activity using other methods (e.g. various biochemical assays both *in vivo* and *in vitro*) are essential to characterize them as biological antioxidants. It may be concluded from this study that *M. quadrifolia* is active against the tested pathogenic microorganisms and also have cytotoxic and antioxidant effects. In addition, the results confirm the use of the plant in traditional medicine. The results of the investigation do not reveal that which chemical compound is responsible for aforementioned activity. Now our next aim is to explore the lead compound liable for aforementioned activity from this plant.

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