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Invitro Cytotoxic Activity of *Marsilea Quadrifolia Linn* of MCF-7 Cells of Human Breast Cancer

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Abstract

The aim of the present study was to evaluate the invitro cytotoxic activity of methanol, aqueous and ethyl acetate extracts of leaves of Marsilea quadrifolia on MCF-7 cells from human breast cancer . Qualitative phytochemical screening tests were performed to detect phytochemicals in the extracts. Antioxidant activity of the plant extract was then characterised using α, α Diphenyl_β- picryl hydrazyl (DPPH) radical scavenging method. Antioxidant activity using DPPH was found to increase, in concentration dependent manner. All the three extracts ethylacetate, aqueous and methanol exhibited potential antioxidant activity with an IC50 value of 10, 125 and 25µg/ml respectively, when compared to the standard BHT with an IC50 value of the 7.5 µg/ml. The cytotoxic activity of the extracts of Marsilea quadrifolia on MCF-7 cells from human breast cancer was investigated invitro 3-(4) 5-Dimethyl-thiazol-zyl)-2,5 biphenyl tetrazolium bromide (MTT). The results showed decreased cell viability and cell growth inhibition in dose dependent manner. The IC50 value of standard 5 fluorouracil, methanol,ethylacetate and aqueous extracts were 9.3, 39.06, 47.82 and 187.5µg/ml respectively. Methanol and ethyl acetate extracts of M.quadrifolia demonstrated strongest antioxidant and anti-proliferative activities. The findings from this study indicated that methanol and ethylacetate extracts of M.quadrifolia leaf possessed vast potential as medicinal drug especially in breast cancer treatment. .

Keywords: Marsilea quadrifolia, MCF-7 cell lines, MTT Assay, Cytotoxic activity, GC-MS, DPPH

Introduction

For centuries, people have been using plants for their therapeutic values. Today 85,000 plants have been documented for therapeutic use globally[1]. Antimicrobial and cytotoxic agents from natural, especially plant sources may be easily accessible and might be cheaper with minimal side effects. The screening of the plant extracts has been of great interest to scientists in the search for new drugs for effective treatment of several diseases[2]. Cancer is one of the most dangerous disease in human's and presently there is considerable scientific discovery of new anticancer agents from natural products. Drug discovery from medicinal plants has played an important role in the treatment of cancer and indeed, over the last half century most of the plant secondary metabolites and their derivation have been used towards combating cancer[3,4]. M.quadrifolia Linn is an aquatic fern belongs to the family (Marsileaceae) commonly named has Aaraikeerai in Tamil, Neeraral in Malayalam and Cauptiya, Sunsuniya in Hindi. It 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. It possess long stalked petiole with 4 clover like lobes and are either held above the water or submerged. Juice made from the leaves is diuretic and febrifuge and also used to treat snake bite and applied to abscesses etc[5]. The plant is anti-inflamatory, diuretic, depurative, febrifuge and refrigerant[6]. Plants pacifies vitiated pitta, cough, bronchitis, diabetes, psychiatric diseases, eye diseases, diarrhea and skin disease. Previous studies have shown that antibacterial cytotoxic and antioxidant activity of crude extract of Marsilea quadrifolia[7]. The present study was performed to characterize the anticancer activity of M.quadrifolia extracts to validate its medicinal potential.

Material and Methods

Plant Material: Fresh plants of M.quadrifolia was collected from Tanjore district of Tamilnadu State, in South India and was authenticated by Prof. P. Jayaraman, Director Institute of Herbal Botany Plant Anatomy Research centre, West Tambaram, Chennai-45, India.

Preparation of Plant Extract: The leaves of M.quadrifolia was washed, shade dried, pulverized in a blender and kept in a air tight container for experimental uses. 20g of dired powder was soaked in 200ml methanol, water and ethylacetate, kept in orbital shaker for 24hrs at 20ºC for 100rpm. The extract was filtered through a cotton plug followed by whatman filter paper no.1 and the extracts were allowed for evaporation. 100mg of the extract was dissolved in 1ml of Dimethyl sulphoxide (DMSO) to prepare the stock solution (100mg/ml).

Phytochemical Screening: Qualitative chemical tests were carried out using extracts from plant to identify the phytochemicals[8,9].

DPPH radical scavenging activity: Free radical scavenging capacity was evaluated on the basis of the scavenging activity of DPPH by measuring the reduction of absorbance at 517 nm[10].

Principle: The DPPH assay measures hydrogen atom (or one electron) donating activity and hence provides a measure of free-radical scavenging antioxidant activity. DPPH is a purple-coloured stable free radical and will form yellow color when it was reduced as diphenyl picryl hydrazine complex.

Crude methanol, aqueous and ethylacetate extracts of M.quadrifolia were redissolved in methanol and various concentrations of each extract were prepared. The assay mixture contained in a total volume of 1 mL consists of 500 µL of the extract, 125 µL of freshly prepared DPPH solution (1mM in methanol) and 375 µL of solvent (methanol). The contents were mixed vigorously in a vortex mixer for 10 s and incubated at room temperature in the dark (wrapped with Aluminum foil) for 30 min. The absorbance was read at 517 nm using a spectrophotometer. In each experiment, the tested sample alone in methanol was used as blank while the DPPH solution alone in methanol was used as control. All experiments were carried out in triplicate. Butylated hydroxy toluene was used as a standard. The radical scavenging activity of samples corresponded to the intensity of quenching DPPH.

The results were expressed as percentage inhibition.
% inhibition = [(A control – A sample)/ A control] x 100
where A control and A sample are the absorbance values of the control and test sample, respectively. The effective concentration of sample required to scavenge DPPH radical by 50% (EC50) was obtained by linear regression analysis of dose-response curve plotting between % inhibition and concentration The EC50 value for each sample, defined as the concentration of the test sample leading to 50% reduction of the initial DPPH concentration, was calculated from the non linear regression curve of Log concentration of the test extract (µg ml-1) against the mean percentage of the radical scavenging activity.

Evaluation of cytotoxic activity: The human Breast adenocarcinoma (MCF-7) cell line was obtained from the NCCS., Pune, India.

The assay was carried out using (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT). MTT is cleaved by mitochondrial enzyme dehydrogenase of viable cells, yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity

Preparation of working herbal extracts: 0.5ml of stock (100 mg/ ml) herbal extract was dissolved in 4.5 ml of DMSO for a concentration of 10 mg/ ml. The fresh working suspension filtered through 0.45 µm membrane filter prior to the assay. Using the 1mg/ ml concentration herbal extract nine serial doubling dilutions of the extract of 500µl each was prepared in DMSO to get the concentration of the extract as indicated and the diluted extracts transferred to 10 wells of a 12 well culture plate. 500 µl of 48h culture of MCF 7 cell lines at a concentration of 105 cells/ ml was added to each well. Two control wells received only cell suspension without plant extract. The plate incubated in a humidified CO_2 incubator at 37°C for 4 - 6 h. The plate was microscopically examined for confluent monolayer of cells, turbidity and toxicity.

MTT assay[11-13]: After incubation, the medium from the wells aspirated carefully and discarded. Each well washed with Eagle's Minimum Essential Medium (EMEM) without Fetal Calf Serum (FCS). 200µl of MTT solution (5mg MTT/ ml of PBS, pH 7.2) added to each well. The plate incubated for 6-7 h at 37°C in a CO_2 incubator with 5% CO_2. After incubation 1ml of DMSO added to each well and mixedwith pipette and left for 45s at room temperature. Purple formazan formed in the wells. Cell control and solvent control were included in each assay to compare the full cell viability in cytotoxicity and antitumor activity assessments. The suspension transferred to a spectrophotometer cuvette and the optical density (OD) measured at 540nm using DMSO as blank. The % cell viability was calculated with the following formula:
Cell viability % = Mean OD of wells receiving each plant extract dilution / Mean OD of control wells x 100.

Results and Discussions

Phytochemical Screening - Qualitative phytochemical screening of the different solventextracts of M.quadrifolia revealed the presence of carbohydrates, alkaloids, steroids, Tannins, Terpenes, Protein, Flavonoids and anthroquinone, absence of Glycosides and Saponin were summarized in the table-1 *Antioxidant Activity* - DPPH is one of the free radical widely used for testing preliminary radical scavenging activity of compound or a plant extract. All the three extracts (methanol, aqueous and ethylacetate) of M.quadrifolia exhibited potential antioxidant activity were depicted in the figure 1.

The ethylacetate extract of M.quadrifolia exhibited 50% DPPH free radical scavenging activity at the lowest inhibitory concentration (IC50 : 10µg/ml). The methanol and aqueous extracts of plant also revealed antioxidant activity with an IC50; 25µg/ml and 125µg/ml respectively, when compared to the standard butylated hydroxyl toluene (7.5µg/ml) were presented in the table 2. These results denotes the presence of antioxidant principles in the extractives.

Cytotoxic activity - Invitro cytotoxic activity of methanol, aqueous and ethylacetate extracts of M. quadrifolia Linn were presented in table 3 and 4. The MTT assay showed an anti-proliferative activity with a IC_{50} value of MCF-7 cells at 39.06 µg/ml of methanolic extracts of M.quadrifolia. Ethylacetate and aqueous extracts of M.quadrifolia showed anti-proliferative activity with an IC_{50} value of 47.825 and 187.5 µg/ml respectively.

Table-1
Phytochemical Screening of Different Extracts of *Marsilea quadrifolia Linn*

S. No.	Phytochemical Constituents	Methanol Extract	Ethylacetate extract	Aqueous extract
1	Carbohydrates	+	+	+
2	Alkaloids	+	+	+
3	Steroids	+	+	+
4	Glycosides	-	-	-
5	Saponin	-	-	-
6	Flavonoids	+	+	+
7	Tannin	+	+	+
8	Protein and amino acids	+	+	+
9	Fats and oils	-	+	-
10	Terpenes	+	+	+
11	Phlobatannin	-	-	-
12	Resin	+	+	+
+ Indicates Positive Test Result, - Indicates Negative Test Result.				

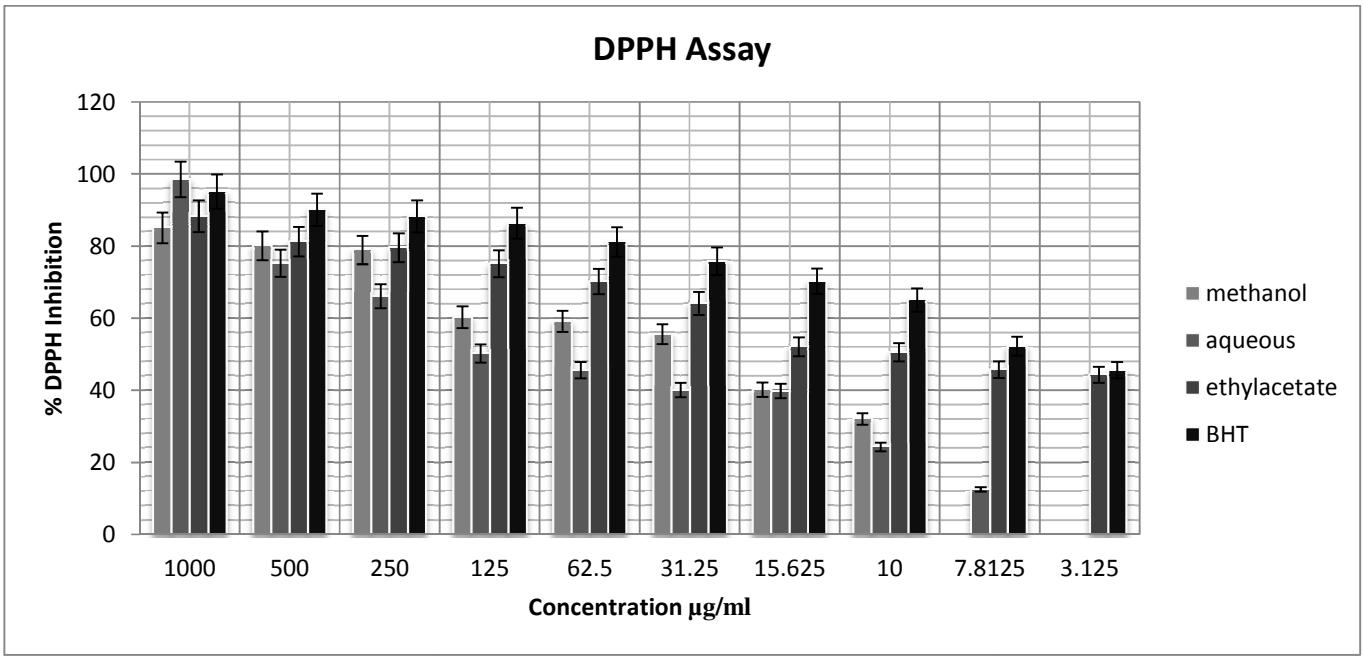

Figure-1
Invitro DPPH Scavenging Activity of Different Extracts of *Marsilea quadrifolia linn*

Table-2
IC 50 value of DPPH Scavenging activity of Different Extracts of *Marsilea quadrifolia*

S. No.	Contents	25 µg/ml
1.	Standard BHT	7.52
2.	Methanol extract	42.40
3.	Ethyl acetate	10.00
4.	Aqueous	125.00

Table-3
Invitro Cytotoxic Activity of Methanol, Ethylacetate and Aqueous Extracts of *Marsilea quadrifolia linn*

S.No	Concentration (µg/ml)	Dilutions	Invitro cytotoxic activity on Breast cancer cell lines (MCF 7 cell line)			5FU (Fluoro uracil)
			Methanol	Aqueous	Ethylacetate	
1	1000	Neat	12.32 ±0.25	32.56 ±0.59	22.58 ±0.55	12.33 ± 0.51
2	500	1:1	15.96 ±0.89	41.23 ±0.32	29.63 ±1.25	17.18 ±1.58
3	250	1:2	18.96 ±1.23	48.92 ±0.57	35.47 ±1.35	20.98 ±1.47
4	125	1:4	25.36 ±0.89	52.35 ±0.44	40.21 ±1.47	24.13 ±0.55
5	62.5	1:8	38.59 ±1.05	59.87 ±0.14	48.96 ±0.25	28.78 ±0.87
6	31.25	1:16	52.55 ±1.47	61.23 ±0.57	51.22 ±0.45	37.17 ±0.69
7	15.625	1:32	58.79 ±1.69	68.77 ±0.22	54.69 ±1.25	43.46 ±0.54
8	10	1:64	65.32 ±0.58	72.02 ±1.06	61.23 ±1.88	48.19 ±1.25
9	7.8125	1:128	78.19 ±0.47	75.12 ±1.08	68.90 ±1.98	54.33 ±1.06
10	3.125	1:256	90.23 ±0.55	85.64 ±1.89	75.88 ±0.58	61.22 ±0.88
11	Cell control	-	100	100	100	100

Table-4
Invitro Cytotoxic Activity Showing IC 50 values of MQ

S. No	Extract	IC 50 Concentration (µg/ml)
1.	Methanol extract	39.06
2.	Aqueous extract	187.5
3.	Ethyl acetate extract	47.825

Conclusion

The results of this study revealed that methanolic extract of M.quadrifolia contains pharmacologically active substances with cytotoxic activity and it showed most effective inhibition of MCF 7 cell proliferation. Accumulating evidence clearly indicates that apoptosis is a critical molecular target by dietary bioactive agents, in the prevention of cancer. Since the phytochemical analysis has shown the presence of potent phytochemicals like alkaloids, phenols, flavonoids, terpenoids, glycosides, saponin, steroids, tannin and sugars etc.Several authors reported that phenolic, acids, flavonoids, steroids, terpenoids are known to be bioactive principles[14,15]. Therefore the crude extracts of M.quadrifolia leaf could be new sources of development of new plant based therapy for management of diseases. Further research is to be carried out to fractionate and purify the extract, in order to find out the molecules responsible for the anti-proliferation activity observed.

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