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Screening of Cytotoxicity Potential of Different Extracts of *Marsileaminuta*

Akhila.S[1*], Bindu A R[2], Rosemary J[2], Bobby S P[2]
1. KVM College of Pharmacy, Kokothamangalam P.O, Cherthala.
2. Department of Pharmaceutical Sciences, Mahatma Gandhi University, Cheruvandoor Campus, Ettumanoor P.O, Kottayam.

ABSTRACT

Cancer, a disease characterized by uncontrolled proliferation of cells that have transformed from the normal cells of the body and is one of the major causes of death in developed and developing nations. Presently available treatment method, chemotherapeutic agents, suffers from drawback of toxicity such as bone marrow suppression, alopecia and vomiting. Its not within the reach of common man. Therefore challenging task is to identify quick and novel methods which can be of therapeutic value in human cancers. Plants have a long history and important role as a source of effective anti-cancer agents. Hence in the present study, one of the traditionally used plant, *M.minuta* was screened for cytotoxicity. Successive solvent extraction was carried out and the petroleum ether, chloroform, ethyl acetate, alcoholic and aqueous extracts *of M.minuta* were prepared. Extracts were subjected to Brine shrimp lethality assay and it was observed that % mortality increased gradually with the increase in concentration of the test samples. Ethyl acetate and alcoholic extracts which showed better cytotoxicty were further screened for their cytotoxicity towards breast cancer cell lines using MTT cell viability assay. In the same, ethyl acetate extract was found to be significantly cytotoxic towards cell lines compared to that of alcoholic extract which may be due to higher amount of phenolic compounds present in them. The results obtained in this study further supported the traditional usage of the drug and the activity of drug can be attributed to major bioactive phytochemical class; poly phenolics, present in the drug.

Keywords: *Marsileaminuta*, cytotoxicity, phenolics, MTT assay, Brine shrimp

*Corresponding Author Email: akhilamadathil84@gmail.com
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INTRODUCTION

Cancer is a disease characterized by uncontrolled proliferation of cells that have transformed from the normal cells of the body. Cancer can invade the adjacent and distant tissues via circulation. The process of cancer development in humans generally takes many years through initiation, promotion and progression. The major causes of cancer are smoking, dietary imbalances, hormones and chronic infections leading to chronic inflammation. Cancer is one of the major causes of death in developed and developing nations. One in three people will be diagnosed with cancer during their life time. Cancer is one of the disease for which effective drugs at affordable prices are not yet available probably due to lack in understanding the cancer pathophysiology. For such a dreadful disease, anticancer drugs have been developed from a variety of sources ranging from natural products (plants and microbes) to synthetic molecules[1]. The widely used drugs are cancer chemotherapeutic agents. They are chemicals that are intended to be toxic for the pathogenic organism (or cancer cells) but innocuous to the host[2]. They suffer from drawback of high toxicity such as bone marrow suppression, alopecia, nausea & vomiting and are not within the reach of commonman. Therefore the challenging task at this moment is to identify the quick and novel methods that can identify and develop molecules, which can be of therapeutic value in human cancers [3]. Plants have a long history of use in the treatment of cancer and have played an important role as a source of effective anti-cancer agents. It is significant that over 60% of currently used anti-cancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms. The search for anti-cancer agents from plant sources started in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins. As a result, the United States National Cancer Institute (NCI) initiated an extensive plant collection program in 1960, focused mainly in temperate regions. This led to the discovery of many novel chemotypes showing a range of cytotoxic activities, including the taxanes and camptothecins[4]**.** Herbs and natural dietary supplements modulate cellular signaling pathways involved in the apoptotic, proliferative, angiogenic processes and metastasis, which are common to many cancers. Besides this, natural products cause the induction of cellular defense detoxifying and antioxidant enzymes which can protect against cellular damage caused by environmental carcinogens or endogenously generated reactive oxygen species. These agents are also known to act against cancer cells by stimulating the natural immune defense present in the body. There is also another exciting role played by these compounds in combination with standard chemotherapeutic agents. They can act as an adjuvant by

lowering the toxicity and enhancing efficacy of standard drugs used in the treatment of more advanced cancers [5]. Marsilea is a genus of approximately 65 species of aquatic ferns of family marsileaceae and in ayurveda, some of these species are recommended for treatment of psycopathy, diarrhea, cough, bronchitis, skin infections and mentioned its toxicity potential[6].Hence, in the present study one of the traditionally used, yet not scientifically investigated, species, *Marsilea minuta*was screened for its cytotoxic activity.

MATERIALS AND METHOD

Extraction of *M.minuta*

Extraction of whole plant of *Marsilea minuta* was carried out using solvents of increasing polarity by hot continuous extraction method using soxhlet apparatus. 200 g of shade dried plant were taken and size reduced, extracted with 1L of petroleum ether in the round bottom flask and extraction was continued for 10 hrs. The extract obtained was collected and concentrated. The concentrated extract was then weighed and stored. Then the residue obtained after extraction was packed into the body of the soxhlet and then extracted with chloroform, ethyl acetate and ethanol in the same manner. Each time before extracting with the next solvent, the drug material was dried at room temperature. Each extract was concentrated by distilling off the solvent and then evaporating to dryness. Finally the marc obtained was packed into a round bottom flask and refluxed with water. The extract obtained was concentrated in vacuum drier to obtain aqueous extract. All the extracts were weighed and stored for further studies[1].

Brine Shrimp Lethality Assay[7]

Hatching Of Brine Shrimp Eggs

Brine shrimp eggs were hatched in sterile artificial sea water prepared from commercial sea salt 40 g/L under constant aeration for 48 hrs under constant aeration in a suitable hatchery. The pH was adjusted to 8.5 using 1N NaOH. The eggs were sprinkled into the apparatus and were suitably illuminated, then incubated at room temperature (30-37°C) for 48 hrs. Newly hatched free-swimming pink-colored nauplii (larvae) were pipetted from the brighter side. The freshly hatched free-swimming nauplii were used for the bioassay.

Sample Preparation

Samples of various extracts were prepared by dissolving in DMSO and then diluted with artificial sea water. Different concentrations that are 10, 50, 100 and 1000 mcg/ml of various extracts of *Marsilea minuta*was prepared.

Method

Ten nauplii were drawn through a glass capillary and placed in each test tube containing 4.5 ml of brine solution. 0.5 ml of the plant extract was added to 4.5 ml of brine solution to adjust the final volume to 5 ml. It was then incubated at room temperature (24-28°C) for 24 hours under the light. Test was performed in triplicate. The numbers of dead nauplii in each well were counted. Analysis of the data was performed by Probit analysis to determine the lethal concentration of half of the test organisms (LC50).

% Mortality = No. of dead or stopped swimming brine shrimp × 100 Total No. of brine shrimps

MTT ASSAY[8,9]

Materials

MCF-7 purchased from NCCS Pune, was maintained in Dulbecco's modified eagles media and grown to confluency at 37°C and 5 % CO_2 in a humidified atmosphere in a CO_2 incubator (NBS Eppendorf, Germany).

Method

The cells were trypsinized (500 µL of 0.025% Trypsin in PBS/ EDTA solution, Hi media) for 2 minutes and passed to T flasks in complete aseptic conditions. Extracts were added to grown cells at a concentration of 100 µg, 500 µg and 1000 µg from a stock of 100 mg/ml and incubated for 24 hours. All assays were done in triplicates and an untreated sample was maintained as negative control and ethanol (10 µL) as positive control. The antiproliferative effect of the samples was determined by MTT cell viability assay. The cell culture suspension was washed with PBS solution and then added with 200 µL MTT solution to the culture (MTT -5mg/volume dissolvedin PBS) and incubated at 37°C for 3 hours. MTT was removed by washing with 1x PBS and 300 µL DMSO to each culture. It was then incubated at room temperature for 30 minutes until all the cells are lysed and a purple colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 minutes to precipitate cell debris. OD was read at 540 nm using DMSO as blank.

% viability = (Mean optical density of test - mean optical density of blank) X 100 (Mean optical density of control - mean optical density of blank)

RESULTS AND DISCUSSION

Medicinal plants constitute a common alternative for cancer prevention and treatment in many countries around the world. Approximately, 60% of the anticancer drugs currently used have been isolated from natural products from the plants. More than 3000 plants worldwide have been reported to possess anticancer properties. Extracts of these medicinal plants are believed to contain

a wide array of phytochemical compounds which might possess cancer preventive and/or therapeutic properties. According to the US NCI plant screening program, a crude extract is generally considered to have *in-vitro* cytotoxic activity if the IC_{50} value (concentration thatcauses reduction in cell viability to 50%) is less than 30 µg/ml. Time and concentration-dependent manner of the extract activities reflects the logicalpharmacokinetics and pharmacodynamics on the cancer cells. This is normally indicated in the cellular uptake across membrane and the metabolic disturbance within the cells. These cellular pathways of activities are concerned with necessary signaling transduction through cytosol and nucleoplasm. The study of drug response and development of drug response model using these cell lines is the key to determine safety and hazardous levels and dosages of the extracts to which the cells areexposed[9].Several studies have shown that brine shrimp assay has been an excellent method for preliminary investigations of toxicity, to screen medicinal plants popularly used for several purposes and for monitoring the isolation a great variety of biologically active compounds. The technique is easily mastered, costs little, and utilizes small amount of test material. Furthermore, a positive correlation between the lethality to brine shrimp and the corresponding oral lethal dose in mice of medicinal plants has been demonstrated[10]. In the present study, evaluation of extracts of *M.minuta* for general toxicity was performed using brine shrimp lethality assay. Different extracts of *Marsilea minuta*; petroleum ether extract, chloroform extract, ethyl acetate extract, alcoholic extracts and aqueous extracts were prepared and examined for their cyotoxicity towards Brine shrimp. Number of Shrimps killed after 24hours were counted and the results obtained after triplicate assay was tabulated in the table 1. Lethal concentrations for all the extracts were calculated using Probit method of analysis and tabulated in the tables 2,3,4,5 and 6 respectively. For all the extracts of *M.minuta*, LC_{50} value were calculated and tabulated in the table 7. In Brine shrimp cytotoxicity assay, it was observed that % mortality increased gradually with the increase in concentration of the test samples. Ethyl acetate and alcoholic extracts which showed better cytotoxicty were further screened for their cytotoxicity towards breast cancer cell lines using MTT assay. Percentage cytotoxicity obtained for ethyl acetate and alcoholic extracts were determined and tabulated in the tables 8 and 9 respectively. IC50 values of ethyl acetate and alcoholic extracts were calculated using Probit analysis and tabulated in the table 10. It was observed that ethyl acetate extract showed moderate cytotoxicity towards breast cancer cell with an IC_{50} value 90.72 mcg/ml where as alcoholic extract showed only mild cytotoxicity against breast cancer cell lines. It may be due to higher amount of phenolic compounds present in the ethyl acetate extract. Traditional usage of plant has been justified from the study results. Recent studies have shown that plant phenolics and flavonoids produce inhibitory

growth in many cancer cell lines such as human breast cancer cells andsolid malignant tumor cells[11]. The anti-tumor activity is highly dependent upon their conformational characteristics, which in turn, determine their antioxidant/pro-oxidantproperties[12,13]. Plant phenolic compounds are able, not only to prevent cancer initiation, progression and metastasis through a variety of cell mechanisms, including immune cell functions and angiogenesis, but are also capable of enhancing standard chemo- and radio therapeutic modalities by reversing the cell mechanisms that lead todesensitization[11]. Hence, the cytotoxicity of ethyl acetate extract of *M.minuta* may be attributed by the plant phenolics as ethyl acetate extract was found to be rich in phenolics and flavonoids compared to other extracts of *M.Minuta*.

Table 1: Observation after 24 hrs

Extract	Total no: of shrimps	No: of shrimps killed out of 10 per dilution after 24hrs				
		Control	10µg/ml	50µg/ml	100µg/ml	1000µg/ml
PE	10	0	0	0.66± 0.57	3.00±0.00	9.33±0.58
CE	10	0	0	1.00± 0.00	4.0±1.00	9.66±0.57
EA	10	0	0	0.66±0.58	3.33±0.58	7.33±0.58
ALC	10	0	0	0.66±0.58	2.66±0.577	7.33±1.54
AQ	10	0	0	0	3.67± 0.57	9.33±1.54

Table 2: Probit value determination:petroleum ether extract

Conc.(mcg/ml)	Log conc.	%Mortality	Corrected %	Probit value
10	1	0	0.833	2.22
50	1.69	0	0.833	2.22
100	2	36.7	36.7	4.65
1000	3	93.33	93.33	6.48

Table 3: Probit value determination: chloroform extract

Conc.(mcg/ml)	Log conc.	%Mortality	Corrected %	Probit value
10	1	0	0.833	2.22
50	1.69	6.6	6.66	3.49
100	2	26.6	26.66	4.37
1000	3	73.3	73.33	5.61

Table 4: Probit value determination: ethyl acetate extract

Conc.(mcg/ml)	Log conc.	%Mortality	Corrected %	Probit value
10	1	0	0.833	2.22
50	1.69	10	10	3.72
100	2	40	40	4.75
1000	3	96.6	96.6	6.83

Table 5: Probit value determination: Alcohol extract

Conc.(mcg/ml)	Log conc.	%Mortality	Corrected %	Probit value
10	1	0	0.833	2.22
50	1.69	6.6	6.66	3.49
100	2	30	30	4.48

1000	3	93.3	93.33	6.48

Table 6: Probit value determination: Aqueous extract

Conc.(mcg/ml)	Log conc.	%Mortality	Corrected %	Probit value
1	1	0	0.833	2.22
50	1.69	6.6	6.66	3.49
100	2	33.33	33.33	4.56
1000	3	73.3	73.33	5.61

Table 7: LC_{50} values of each extract of *M.minuta*

Extracts	LC_{50} value(mcg/ml)
PE	254.67
CE	361.54
EA	155.38
ALC	204.83
AQ	337.12

Table 8: Percentage cytotoxicity of ethyl acetate extract

Conc.(µg/ml)	Log conc.	Absorbance	% Viability	% Cytotoxicity	Probit
100	2	0.410±0.032	49.1	50.9	5.027
500	2.69	0.328± 0.013	39.32	60.68	5.27
1000	3	0.272± 0.044	32.61	67.39	5.44

Table 9: Percentage cytotoxicity of alcoholic extract

Conc.(µg/ml)	Log conc.	Absorbance	%Viability	% Cytotoxicity	Probit
100	2	0.213±0.003	58.19	41.81	4.794
500	2.69	0.109±0.025	29.78	70.72	5.52
1000	3	0.096±0.001	26.22	73.78	5.631

Table 10: $IC_{50}$of ethyl acetate and alcoholic extract of *M.minuta*

Extracts	IC_{50} value(mcg/ml)
EA	**90.72**
ALC	173.4

CONCLUSION

Ethyl acetate extract and alcoholic extract with maximum amount of phenolics and flavonoids showed significant and better cytotoxic activity when compared to other extracts of *M.minuta*. Also, significant cytotoxicity against breast cancer cell lines was evident for the ethyl acetate extract of *M.minuta*. The results obtained in this study further supported the traditional usage of the drug and the activity of drug can be attributed to major bioactive phytochemical class; poly phenolics, present in the drug. Moreover, the detailed phytochemical profiling of ethyl acetate extract of *M.minuta* might be corner stone to obtain new anticancer agents.

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