An Elsevier Indexed Journal ISSN-2230-7346

Journal of Global Trends in Pharmaceutical Sciences

EVALUATING *IN VITRO* ANTIOXIDANT ACTIVITY AND LC-MS ANALYSIS OF *MARSELIA QUADRIFOLIA*.

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ARTICLE INFO

Key Words
Marsilea quadrifolia, LCMS, Superoxide, Peroxidase.

ABTRACT

The antioxidant activity was assayed in the aqueous, ethanol, methanol and ethyl acetate extract of *Marsilea quadrifolia*. In this study significant free radical scavenging activity was determined by evaluating the inhibition concentration in each test. Superoxide scavenging and Peroxidase assay shows better activity in the methanol extract, highest activity of Catalase was observed in the aqueous extract and reducing power assay shows higher activity in the ethyl acetate extract of the plant. It was evaluated that antioxidant activity was found to be high in the methanol extract, hence the LC-MS study was performed in the methanol extract of the plant In this study, the bioactive components of the methanolic extract of the whole plant *Marsilea quadrifolia* have been evaluated using LC-MS, it showed the presence of 6 different phytochemical compounds like 9,12 octodeca dienoic acid with molecular weight of 280, didodecylpthalate with molecular weight of 338, 1,2 benzene dicarboxylic acid, diisoctyl ester with molecular weight of 390, 4,8,12,16-Octadecatetraen-1-ol, 4,9,13,17-tetramethyl- with molecular cumulative effect of the phytochemicals present in the plant which genuinely designate them as free radical scavenger.

INTRODUCTION

Free radicals play a dual role as they can be either harmful or helpful to the body (Pham-Huy *et al.*, 2008). So, it will be appropriate to examine the possible role of free radicals in disease and most importantly, harnessing the therapeutic

phytochemicals from *Marsilea quadrifolia* against these pro-oxidants. It is well documented that a number of physiological processes in human body lead to the generation of a series of oxygen-centered free radicals namely reactive oxygen species (ROS) and reactive nitrogen species (RNS) as by-products. However, imbalance in their production impairs the innate antioxidant defense system of the cell, resulting in peroxidation of unsaturated fatty acids, membrane protein damage (proteins, carbohydrates denaturation) and DNA mutation (nucleic acids denaturation) causing oxidative/nitrosative stress (Maes *et al.*, 2011) which ultimately initiate the genesis of many multifactorial diseases. While the use of synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Propylgallate (PG) and butylated hydroquinone have often been implicated to achieve immediate result, recent data indicates that these synthetic antioxidants could have carcinogenic effects thus fueling an intense search for newer and efficient antioxidants (Yevgenia *et al.*, 2013).

Plants have been used as a major source of medicines (Prabuseenivasan *et al.*, 2006 and Chen *et al.*, 2008). The Indian ancient medicinal therapies like Ayurveda, list majority of the plants found in India. The detailed applications of these plants in herbal medicines have been mentioned as Ayurveda (Pavithra *et al.*, 2010). *M. quadrifolia* is an aquatic fern which belongs to the family Marsileacea, which is commonly called European Water clover. The methanolic extract of *M. quadrifolia* contains majorly steroids and antioxidant property which may responsible for anti amnesic effect (Ashwini *et al.*, 2012). Taking into consideration of the medicinal importance of *M. quadrifolia* the methanol extract of whole plant of *M. quadrifolia* were analyzed using LC-MS.

MATERIALS AND METHOD

COLLECTION OF PLANT SAMPLE

The plant *M. quadrifolia* was collected from Kanya Kumari district, Tamil Nadu, India. The plant was then identified by the book "The flora of Presidency of Madras" (Gamble, 1958). The whole plant were cleaned and wet dried for three weeks and grounded into a fine powder, which was used for further extraction.

Preparation of plant extract

Crude plant extract was prepared by Soxhlet extraction method. The powdered plant materials was extracted with aqueous, ethanol, methanol, and ethyl acetate at 40 - 80˚C depending upon the evaporation point of the solvent by Soxhlet extraction. The extraction was carried out using solvent of increasing polarity from ethyl acetate, ethanol, methanol and water respectively. The process of extraction continues for 24 hours or till the solvent in siphon tube of an extractor become colourless. After that, the extract was taken in a beaker, kept on hot plate and heated at 30 - 40ºC till all the solvent got evapourated. Dried extract was kept in refrigerator at 4ºC for further use.

ANTIOXIDANT ASSAYS

Catalase assay

To 0.5 ml of enzyme extract add the reaction mixture containing 1ml of 0.01 M phosphate buffer (pH 7.0), 0.5 ml of 0.2 M H_2O_2, 0.4 ml H_2O and incubated for

different time period. The reaction was terminated by the addition of 2 ml of acid reagent (dichromate/acetic acid mixture) which was prepared by mixing 5% potassium dichromate with glacial acetic acid (1:3). To the control, the enzyme was added after the addition of acid reagent. All the tubes were heated for 10 minutes and the absorbance was read at 610 nm (Sinha, 1972).

Peroxidase assay

To the reaction mixture (consist of 3ml of buffered pyrogallol [0.05 M pyrogallol in 0.1 M phosphate buffer (pH 7.0) and 0.5ml of 1% H_2O_2) add 0.1 ml of enzyme extract. Absorbance was measured at 430nm for every 30seconds for 2minutes. The peroxidase activity was calculated using an extinction coefficient of oxidized pyrogallol (4.5Litres/mol) (Addy and Goodman, 1972).

Reducing Power Assay

A spectrophotometric method (Ferreira *et al.*, 2007) was used for the measurement of reducing power. For this 2.5ml of each of the extracts was mixed with 2.5ml phosphate buffer (0.2M, pH 6.6) and 2.5ml of 1% potassium ferricyanide (10mg/ml). the mixture was incubated at 50ºC for 20min, then rapidly cooled, mixed with 2.5ml of 10% trichloroacetic acid and centrifuged at 650rpm for 10min. an aliquot (2.5ml) of the supernatant was diluted with water (2.5ml) and ferric chloride (0.5ml, 0.1%) was added and allowed to stand for 10min. the absorbance was read spectrophotometrically at 700nm. A higher absorbance indicates a higher reducing power, Fe^{2+} chelating property.

Superoxide Radical Scavenging Activity

The superoxide free radical scavenging activity of the test extracts was determined by the method of Mccord and Fridovich which depends on the light induced super oxide generation by riboflavin and the corresponding reaction of nitro blue tetrazolium (NBT).

The assay mixture contained different concentrations of test extracts and EDTA (6µM containing 3µg of NaCN, NBT (50µM), riboflavin (2µM) and phosphate buffer (58mM, pH 7.8) to give a total volume of 3ml. The tubes are uniformly illuminated for 15min and there after the absorbance are measured at 560 nm. The percentages inhibition by the test drug of superoxide production was evaluated by comparing the absorbance values of standard and experimental tubes. Gallic acid in distilled water at different concentrations is used as standard for comparison.

LCMS ANALYSIS

For Multi stage mass spectrometer (MS^n) experiments, LTQ-XL-MS 2.5.0 instrument (Thermo, CA, USA) was used. The mass spectra were acquired and processed using Xcalibur software (version 2.0). The mass parameters used were: mass range, 50-1000 amu; vaporizer temperature, 200 °C; helium gas flow rate (used to improve the ion trapping efficiency), 0.5 ml/min; scan rate for product ions, 11000 amu/s; and sample infusion flow rate, 10 µl/min.

Various experiments were performed with analyte extracted in different solvents finally after screening of various extracts better results were obtained with methanolic extract. In other extracts droplet formation and ionisation

efficiency is reduced and sub sequentially leads to precipitation of analyte in ionisation source leads to contamination in source. But with methanolic extracts output was optimum with better intensity. Experiments were done in Electronic Spray Ionisation (ESI) +Ve mode.

RESULT AND DISCUSSION

Superoxide anion is one of the most representative free radicals. In cellular oxidation reactions, superoxide radicals have their initial effects magnified because they produce other kinds of cell-damaging free radicals, hydroxyl radicals and oxidizing agents (Halliwell and Gutteridge, 1999). Comparing the aqueous, ethanol, methanol and ethyl acetate extract of *Marsilea quadrifolia* it shows methanol extract has higher scavenging level of 29.62±0.03, following this aqueous extract shows 28.43±0.05, ethanol 22.13±0.04 and ethyl acetate 13.42±0.062 as its superoxide scavenging activity.

The reducing ability of a compound generally depends on the presence of reductants (Duh *et al.,* 1999) which have been exhibiting antioxidative potential by breaking the free radical chain and donating a hydrogen atom (Gordon, 1990). The presence of deductants in the aqueous, ethanol, methanol and ethyl acetate extract causes the reduction of the Fe^{3+}/ferricynide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Purssian blue at 700nm. Table.1 shows the reducing power of *M. quadrifolia* ranges from ethyl acetate extract 19.01±0.51; methanol extract 17.04±0.05, aqueous extract 14.06±0.09 and ethanol extract 10.51±0.04 levels. Superoxide scavenging activity was high in methanol extract and low in ethyl acetate extract. The reducing power assay was high in ethyl acetate extract and lower in ethanol extract.

Table 1: Superoxide scavenging activity and reducing power assay for aqueous, ethanol, methanol and ethyl acetate extract of *M. quadrifolia*

Assays	Aqueous extract	Ethanol extract	Methanol extract	Ethyl acetate extract
Superoxide scavenging	28.43 ±0.05	22.13± 00.04	29.62 ±0.03	13.42± 0.062
Reducing power	14.06 ±0.09	10.51± 0.04	17.04 ±0.05	19.01± 0.51

Each value represent the mean ± SD n = 3

The activity of Catalase was increased significantly with increased time interval in the aqueous, ethanol, methanol and ethyl acetate extract of *M. quadrifolia*. Highest activity of Catalase was observed in aqueous extract by 31.48±0.13 in 0 sec, 33.3±0.14 in 30sec, 35.85±0.13 in 60sec and 36.7±0.13 shown in Fig 1, followed by this methanol extract with 13.17±0.79 in 0sec, 16.15±0.7 in 30sec, 18.23±0.8 in 60sec and 22.98±0.87 in 90sec, then ethanol extract with 6.29±0.9 in 0sec, 8.01±0.91 in 30sec, 8.87±0.86 in 60sec and 11.35±0.9 in 90sec and the lowest activity was found in the ethyl acetate

extract with 2.54±0.9 in 0sec, 3.48±0.93 in 30sec, 3.82±0.84 in 60sec and 4.22±0.94 in 90sec. Highest Catalase activity depends on extractability, stability and properties of the crude enzyme. Maximum activity has been recovered due to extraction of more protein and optimum temperatures ranging from 5°C to 55°C (Beulah and Ramana, 2013).

Peroxidase activity was high in methanol extract of the plant *M. quadrifolia* with 4.9±0.05 in 0 sec, 4.98±0.03 in 30secs, 5.43±0.04 in 60secs and 5.48±0.06 in 90secs, following by this ethyl acetate extract with 2.68±0.05 in 0sec, 3.31±0.03 in 30secs, 3.67±0.04 in 60secs and 4.03±0.05 in 90secs, then aqueous extract with 2.12±0.02 in 0sec, 2.23±0.01 in 30secs, 2.53±0.01 in 60secs and 2.77±0.01 in 90secs and the lowest peroxidase activity was found in the ethanol extract with 1.52±0.04 in 0sec, 1.64±0.03 in 30secs, 1.82±0.04 in 60secs and 2.04±0.03 in 90secs (Fig 1). Plant peroxidases are monomeric heme containing enzymes (Siegel, 1993). Peroxidase reduces hydrogen peroxide to water while oxidizing a variety of substrates. Thus, peroxidases are oxidoreductases which use hydrogen peroxide as electron acceptor for catalyzing different oxidative reactions (Gacche *et al.*, 2010)**.**

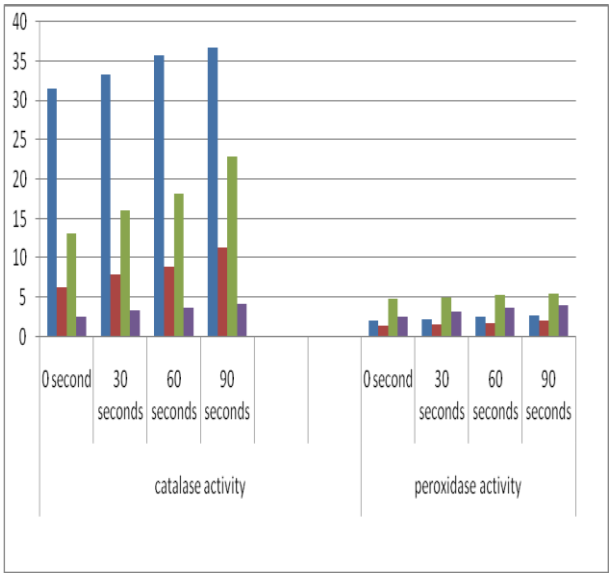

Fig. 1 Catalase and Peroxidase activity of the extracts of the plant *M. quadrifolia*

LC-MS Analysis of Bioactive Constituents

The compounds present in the methanol extract of whole plant of *M. quadrifolia* were identified by LC-MS analysis (Fig. 2). The active principles with their molecular formula, molecular weight (MW) and molecular formula in the methanol extract of whole plant of *M. quadrifolia* are presented (Table 2). The prevailing compounds in methanol extract of whole plant were 9,12 octodeca dienoic acid with molecular weight of 280, didodecylpthalate with molecular weight of 338, 1,2 benzene dicarboxylic acid, diisoctyl ester with molecular weight of 390, 4,8,12,16-Octadecatetraen-1-ol, 4,9,13,17-tetramethyl- with molecular weight of 502, Chlorogenic acid with molecular weight of 354 and 5-Stigmastan-3, 6-dione with molecular weight of 428.

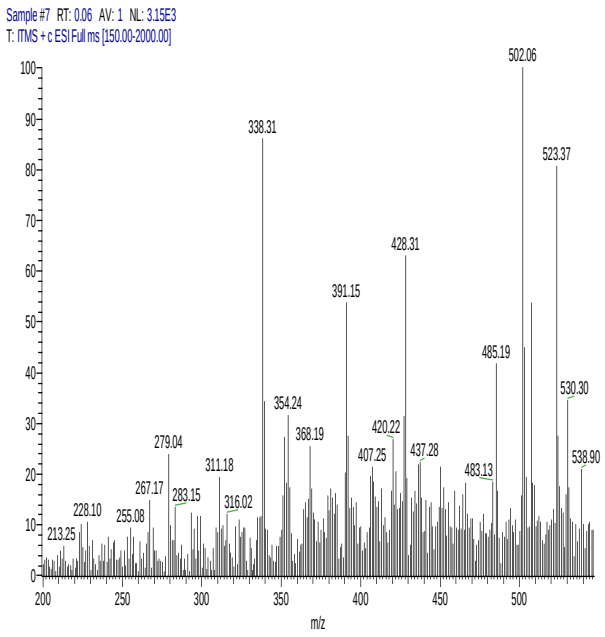

Fig. 2. LC-MS spectrums of methanol extract of the plant *M. quadrifolia*

The compounds identified by LC-MS analysis with its molecular formula, molecular weight, its activity and the nature of compounds were tabulated in Table. 2.

Table. 2. LC-MS analyses of the methanol extract of the plant *M. quadrifolia*

S. No	Name of Compound	Molecular Formula	Molecular Weight	Activity	Nature Of Compound
1	9,12 octodecadienoic acid	$C_{18}H_{32}O_2$	280	Hypocholesterolemic Nematicide Antiarthritic, Hepatoprotective Anti androgenic Nematicide 5-Alpha reductase inhibitor, Antihistamin ic Anticoronary Insectifuge Antieczemic Antiacne Anticancer	Linoleic acid ester
2	didodecyl pthalate	$C_{32}H_{54}O_4$	338	Antimicrobial, Antifouling	Plasticizer compound
3	1,2 benzene dicarboxylic acid, diisoctyl ester	$C_{24}H_{38}O_4$	390	Antimicrobial, Antifouling	Plasticizer compound
4	4,8,12,16-Octadecatetraen-1-ol, 4,9,13,17-tetramethyl-	$C_{32}H_{54}O_4$	502	Antimicrobial	Unsaturated alcohol compound
5	Chlorogenic acid	$C_{21}H_{38}O_4$	354	Dietary supplement, reduce blood pressure, anti-inflammatory, for respiratory allergies	Ester
6	5-Stigmastan-3, 6-dione	$C_{29}H_{48}O_2$	428	Reduce blood level of glucose, hypercholesterolemia	Phytosterols

The lipids present in *M. quadrifolia* contain relatively large amount of gammalinolenic acid (GLA), an omega-6 (18:3, n-6) fatty acid (all cis-6, 9, 12 octadecatrienoic acid) which contains the first double bond at 6th carbon atom from the methyl (ω) end of the fatty acid chain. GLA is rapidly converted to dihomogammalinolenic acid (DGLA) (20:3, n-6) (a precursor of

antiinflammatory prostaglandin E1) which competes with arachidonate for oxidative enzymes thereby reducing production of cyclooxygenase products derived from arachidonate. In addition, DGLA is converted by 5-lipoxygenase to 15-hydroxy DGLA which possesses 5-lipoxygenase inhibitory activity (Ziboh and Chapkin, 1987). The results of the different studies shows that linolenic acid could inhibit both cyclooxygenase and lipoxygenase pathways of inflammation (dual inhibitory property) (Singh and Majumdar, 1999).

Chlorogenic acid is also a bioflavonoid which exhibits the phamacological activity like antioxidant, antidiabetic and anti obesity (Parul *et al.*, 2007). Stigmastane-3, 6-dione has anti-inflammatory activity (Okoye *et al.*, 2008), 5 alpha-Stigmastane-3,6-dione shows antifeedant activity against bell weevils (Miles *et al.*, 1991).

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Linolenic acid, a - 3 (18:3, n-3) fatty acid (allcis-9, 12, 15 octadecatrienoic acid), is progressively metabolized in the body to 6, 9, 12, 15octadecatetraenoic acid (18:4, n-3), stearadonic acid (20:4, n-3) and eicosapentaenoic acid (20:5, n-3). The end product, eicosapentaenoic acid, has the capacity to competitively inhibit the formation of prostaglandins and leukotrienes derived from arachidonate while serving as a substrate for synthesis of prostaglandins with three double bonds and leukotrienes with five double bonds, which are anti-inflammatory. This could be possible mechanism for the antiinflammatory activity of linolenic acid (Lee *et al.*, 1984).

CONCLUSION

Thus summarizing these results, “it is evident that methanol extract of *M. quadrifolia* proved to have superior antioxidant capacity when compared to aqueous, ethanol and ethyl acetate extracts in this particular study and this may have resulted due to the greater extraction capacity of methanol when used as solvent. Hence, the possibility of using a crude extract as an antioxidant would greatly reduce the need to obtain pure compounds via expensive industrial purification techniques. Further in depth toxicity and dosage may reveal its efficacy of the plant as an alternative to anti-oxidant therapy.

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