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Research Article

QUANTITATIVE PHYTOCHEMICAL ANALYSIS, *IN VITRO* ANTIOXIDANT POTENTIAL AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY STUDIES IN ETHANOLIC EXTRACT OF *AZOLLA MICROPHYLLA*

KUNNATHUPARA BHASKARAN SREENATH, SOWMYA SUNDARAM, VELLIYUR KANNIAPPAN GOPALAKRISHNAN, KANNAPPAN POORNIMA*

Department of Biochemistry, Karpagam Academy of Higher Education, Coimbatore - 641 021, Tamil Nadu, India.
Email: poornimabiochem@karpagamuniversity.edu.in

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ABSTRACT

Objective: The study was intended to analyze phytochemicals quantitatively, evaluate *in vitro* antioxidant properties and to determine the bioactive compounds in the crude extract of *Azolla microphylla* (AM) available at the local farms of Coimbatore district in Tamil Nadu.

Methods: The quantitative phytochemical and *in vitro* antioxidant analyses were performed using standard procedures. The bioactive compounds were analyzed using gas chromatography-mass spectrometry (GC-MS) instrument.

Results: The quantitative phytochemical analysis of AM revealed the presence of considerable amounts of phenols (90.2±2.85 mg gallic acid equivalents/g), tannins (82.2±5.25 mg tannic acid equivalents/g), flavonoids (58.5±1.87 mg quercetin equivalents/g), saponins (12.1±3.78 mg/g), and alkaloids (2.2±0.55 mg/g) in decreasing order of concentrations. The *in vitro* antioxidant analyses suggested that the whole plant extract of AM has prominent antioxidant prospective against various free radicals such as 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azinobis-3-ethylbenzothialozine-6-sulphonicacid, nitric oxide, superoxide, and ferric ions while ascorbic acid being the standard antioxidant used. The GC-MS analysis displayed the presence of 21 bioactive compounds, each belonging to various categories of phytochemicals such as chalcones, terpenoids, fatty acids, coumarins, and steroids.

Conclusion: The results indicate that AM present in the local farms of Coimbatore is an effective scavenger of free radicals and has the potential to be used as a natural antioxidant which is attributed to the rich presence of secondary metabolites.

Keywords: *Azolla microphylla*, Chalcones, Coumarins, Saponins.

INTRODUCTION

Modern day food and lifestyle have resulted in an increasing number of diseases and disorders. A major percentage of the population in developing countries depends on herbal medicines for their primary health care needs [1]. Apparently, the interest for the search and development of new herbal-based drugs are gaining much attraction, as they contain potential bioactive compounds that are more systemic and easily biodegradable [2]. A sufficient number of plants have been proven to be effective against various ailments and massively screened for their therapeutic compounds. Since ancient times, plants belonging to the division Pteridophyta have been known for its medicinal and therapeutic ability. Many species of plants in this division are determined to have potential secondary metabolites that act against many diseases [3]. *Azolla* is an aquatic pteridophyte, which lives in a symbiotic relationship with the blue-green alga, *Anabaena azollae* (AM). Besides being used a biofertilizer, much research interest is developing toward the identification of the medicinal values of *Azolla* species. Literature study reveals that the plant possesses antimicrobial [4], hepatoprotective [5] and antiulcer effects [6]. The above-mentioned properties of the plant could be credited to the presence of various primary and secondary metabolites in significant quantity. In general, the nutrient and phytochemical composition of aquatic macrophytes could vary depending on the season, the place, the soil and the morphology of the plants [7].

Although previous reports on *Azolla* species have observed appreciable results, there exists still a paucity of information with respect to quantification of secondary metabolites and GC-MS analysis in the ethanolic fraction of AM available at the local farms of Coimbatore. The results of the study would definitely benefit the local population of this district and hence, an attempt has been made to investigate the presence of secondary metabolites (quantitative), *in vitro* antioxidant properties and identification of bioactive compounds through gas chromatography-mass spectrometry (GC-MS) analysis.

METHODS

Extracts preparation and phytochemical screening

Fresh samples of AM were collected from the local farms of Coimbatore district and authenticated by Dr. G. V. S. Murthy from TNAU, Coimbatore (Voucher#BSI/SRC/5/23/2014-15/Tech/623). The collected plant materials were washed gently with tap water, rinsed with distilled water and kept for air drying. Air-dried samples were grounded to a fine powder. Pre-weighed plant powder (20 g/200 ml) was extracted sequentially with five different solvents such as petroleum ether, chloroform, ethyl acetate, ethanol and aqueous using Soxhlet apparatus for 8 hrs to extract the polar and non-polar compounds [8]. The extracts obtained were evaporated to dryness using a rotary evaporator at 40°C. Subsequently, dried extracts were refrigerated until further use. Qualitative phytochemical screening of different metabolites was carried out using the methods described previously [9,10].

Quantification of total alkaloids

A pre-weighed quantity (5 g) of AM powder was added to a 250 ml conical flask. Into this, added 200 ml of acetic acid (10%) in ethanol and allowed to stand for 4 hrs. The solution was filtered and concentrated to one-fourth of its original volume using a water bath maintained at 80°C. Concentrated ammonium hydroxide was added in drops to the filtered solution until the precipitation was complete. Later, the entire solution

was settled, filtered and the residue obtained was weighed. The amount of alkaloids was expressed as mg/g of the sample [11].

Quantification of total phenols

About 100 µl of plant extract was mixed with distilled water and Folin–Ciocalteu reagent, and the volume was made up to 3 ml. The mixture was added with sodium carbonate (20%) and incubated in a dark room for 30-40 minutes after gentle mixing. The absorbance was measured at 725 nm using a spectrophotometer against a reagent blank. A standard calibration curve was constructed using different concentrations (30-150 µg/ml) of gallic acid and the total phenolic content was expressed as mg gallic acid equivalent/g [12].

Quantification of total flavonoids

The total flavonoids content was determined by spectrophotometric methods as described previously [13]. To 0.5 ml of the extract, methanol (30%), sodium nitrite and aluminum chloride was added and mixed well. After 5 minutes incubation, 1 ml of sodium hydroxide was added, and the absorbance was measured at 506 nm. A standard calibration curve was constructed using different concentrations (20-100 µg/ml) of quercetin and the total flavonoids content was expressed as mg quercetin equivalent/g.

Quantification of total tannins

To a known quantity of plant extract (0.1 g) and standards (0-100 ppm), distilled water (20 ml), sodium carbonate (15%) and 2.5 ml of Folin–Denis reagent were added. After mixing thoroughly, the reagent containing tubes were incubated at room temperature for 20 minutes and the bluish-green color developed was read at 700 nm. The amount of tannin present in the extract was calculated from the standard curve, and the result was expressed as mg tannic acid equivalent/g [14].

Quantification of total saponins

Total saponins were estimated using gravimetric methods of Obadoni and Ochuko [11]. After the addition of ethanol to a known amount of plant powder, the conical flask was shaken well and kept for 5 hrs in a rotary shaker maintained at 55°C. After filtration, the residue was re-extracted with ethanol and the extracts were pooled. The volume of the pooled extracts was reduced to 50 ml using a water bath set for 90°C. The concentrate was mixed with diethyl ether in a separating funnel and shaken vigorously. The aqueous layer was recovered, while the ether layer was discarded. Repeated the process thrice and butanol was added finally to the pooled extracts. The total saponins content was expressed as mg/g sample.

***In vitro* free radical scavenging assays**

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Various concentrations of the sample and ascorbic acid standards were pipetted out into different test tubes. Methanol was used to make up the volume to 0.5 ml. To these tubes, 4 ml of DPPH solution 0.1 mM was added and mixed well and incubated in a dark room for 15-20 minutes. The absorbance was read at 517 nm against a blank solution. Ascorbic acid in the concentration of 0-100 µg/ml was used as a standard antioxidant. The percentage inhibitions versus concentrations were plotted, and the IC_{50} value was calculated for the sample and standard [15]. Using the below-mentioned equation the percentage inhibition was calculated:

$$\% \text{ Inhibition} = \frac{\text{Abs Control - Abs Sample}}{\text{Abs Control}} \times 100$$

where Abs is absorbance.

2,2'-azinobis-3-ethylbenzothialozine-6-sulphonicacid (ABTS) radical scavenging activity

The extent of the plant extract to scavenge the ABTS radical cation was experimented using the method of Siddhuraju and Manian [16] with minor modifications. An equal volume of 7 mM concentration of ABTS was mixed with 2.45 mM ammonium per sulfate, and the mixture was allowed to stand in the dark at room temperature for 12-16 hrs before use. The reaction was started by the addition of 1 ml of the diluted ABTS solution to different concentrations of ethanolic extract. Ascorbic acid in the concentration range of 0-100 µg/ml was used as standard antioxidant. After 10 minutes, the decrease in the absorbance was measured at 730 nm. The percentage inhibition was calculated using the aforementioned equation in DPPH assay.

Nitric oxide (NO) radical scavenging activity

The principle of this assay is based on the ability of the plant extract to scavenge NO molecules produced in the aqueous solution of sodium nitroprusside at physiological pH. To different concentrations of the plant extract, sodium nitroprusside and phosphate buffered saline (pH = 7.4) was added. The reaction mixture was mixed well and incubated for 15 minutes. The absorbance values were noted at 546 nm after the addition of Griess reagent [17]. The percentage inhibition was calculated using the same equation as DPPH assay. Ascorbic acid was used as a standard reference.

Superoxide (SO) radical scavenging assay

The SO radical scavenging activity of AM extract was assessed by nitro blue tetrazolium (NBT) method described previously by Fontana *et al.* [18] with minor modifications. Briefly, 3 ml of tris-HCl buffer (pH=8), 1 ml of NBT (50 µM) solution, 1 ml of reduced nicotinamide adenine dinucleotide solution was mixed with various concentrations of the plant extract and standard ascorbic acid. The reaction was initiated by the addition of phenazine methosulfate solution to the mixture. After 20 minutes of incubation, the absorbance was read at 560 nm against a reagent blank. Percentage inhibition was calculated using the below-mentioned equation:

$$\% \text{ Inhibition} = \frac{\text{1-Abs Sample}}{\text{Abs Control}} \times 100$$

where Abs is absorbance.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay determines the total reducing potential of the plant extract. FRAP reagent was freshly prepared by adding 3 ml of 2, 4, 6-tripyridyl-s-triazine, 3 ml ferric chloride solution and 24 ml acetate buffer. To the known volume of FRAP reagent, 100 µl of water and 50 µl of varied concentrations of the plant extract was added and mixed well. The increase in the absorbance following the reduction of Fe^{3+} to Fe^{2+} was measured at 593 nm. Ascorbic acid was used as standard reference. The result was expressed as mmol of Fe (II) equivalent/mg sample [19].

GC-MS analysis

Based on the preliminary phytochemical results, ethanolic extract of AM was chosen for the analysis of possible bioactive compounds by GC and MS technique. The Trace GC Ultra (GC) and DSQII (MS) model were purchased from Thermo Fisher Scientific Limited. The GC equipment was fitted with a capillary standard non-polar column with the following dimensions: 30 m*0.25 mm*0.25 µm (length*inner diameter*film). The GC was interfaced to a mass selective detector which computes the mass of compounds through XCALIBUR software. The total run time was 40.51 minutes. The following temperature profiles were used for GC-MS analysis: Injector port and interface (250°C), Ion source (200°C), Oven temperature (80°C to 200°C at the rate of 6°C/minutes). The MS instrument was set to scan in the range between 50 and 650 Da. A total sample volume 1 µl was injected. With a flow rate of 1 ml/minute, helium was used as a carrier gas.

The mass spectrum of compounds was interpreted using NIST4 (National Institute of Standard and Technology) and WILEY9 databases. Each database contains more than five million reference compounds and those compounds with a spectral fit value equal to, or greater than 700 were considered positive [20,21].

Statistical analysis

The data are expressed as a mean±standard deviation from three independent experiments except for GC-MS analysis. The statistical

analysis and IC_{50} values were calculated using Microsoft excel software (2007) for Windows operating system.

RESULTS

Quantitative analysis of secondary metabolites

The analysis of secondary metabolites in plants is essential for the extraction, isolation, identification and purification of various drug metabolites. The results of the qualitative analysis indicated the presence of phenols, flavonoids, alkaloids, steroids, tannins, cardio glycosides, and saponins only in the ethanolic extract of AM. Hence, this extract was used for the further studies. The results of the quantitative analysis indicated the presence of significant quantities of phenols (90.2±2.85 mg/g), tannins (82.2±5.25 mg/g), flavonoids (58.5.2±1.87 mg/g), saponins (12.1±3.78 mg/g), and alkaloids (2.2±0.55 mg/g) in descending orders of concentration Table 1.

In vitro antioxidant activities

The *in vitro* antioxidant activities of ethanolic extract of AM were tested using DPPH, ABTS, NO, SO and FRAP assays. The scavenging activities of standard antioxidant (ascorbic acid) were also studied concomitantly with the plant extract, and their corresponding IC_{50} values are shown in Figs. 1-5.

GC-MS analysis

The GC-MS chromatogram of the ethanolic extract of AM is displayed in Fig. 6. GC-MS analysis resulted in the identification of 21 different compounds. Their retention time, molecular formula and the area % (area percentage) are presented in Table 2.

Table 1: Quantitative analysis of secondary metabolites in *Azolla microphylla*

Parameters	Contents
Total phenols (mg/g of GAE)	90.2±2.85
Total tannins (mg/g of TAE)	82.2±5.25
Total flavonoids (mg/g of QE)	58.5±1.87
Total saponins (mg/g sample)	12.1±3.78
Total alkaloids (mg/g sample)	2.2±0.55

Values are expressed as mean±SD for three determinations. SD: Standard deviation, GAE: Gallic acid equivalent, TAE: Tannic acid equivalent, QE: Quercetin equivalent

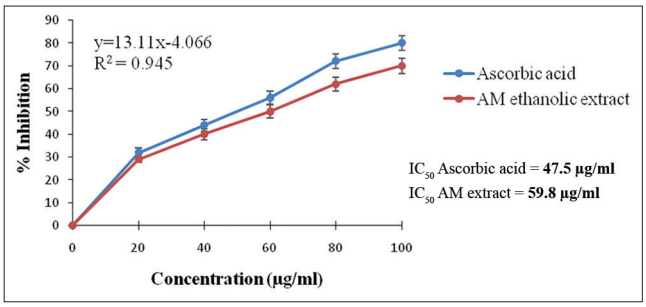

Fig. 1: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

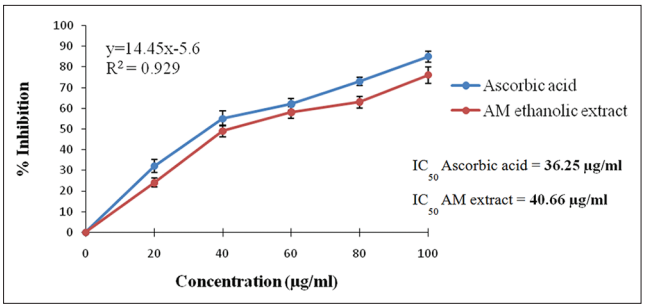

Fig. 2: 2,2'-azinobis-3-ethylbenzothialozine-6-sulphonicacid radical scavenging activity

DISCUSSION

Aquatic plants have economic and environmental importance depending on their chemical composition. Accordingly, some are used for human consumption while the others are used for medicinal values [22]. The pharmacological potential of any plants is dependent on the composition of secondary metabolites, which is unique for the individual taxa [23].

Plant derived secondary metabolites such as alkaloids, polyphenols, saponins, and steroids are gaining much attention in recent years due to their imperative medicinal activities like antioxidant, anti-tumor, anti-diabetic, etc. The screening of aquatic plants to identify the potential antioxidant activities is increasing due its significant concern for safe, lesser side effects and ease of cultivation [24].

The results of the present study suggest that the ethanolic extract of AM contains considerable quantities of phenols, tannins and flavonoids in a decreasing order of concentrations. However, some minor quantities of alkaloids and saponins were also detected. The higher amounts of phenols are essential for the regulation of plant growth and disease resistance, whereas, plants rich in flavonoids are used as natural antioxidants with reported effects such as anti-mutagenic, anti-carcinogenic, and cytoprotective. [25]. Tannins are known to elicit

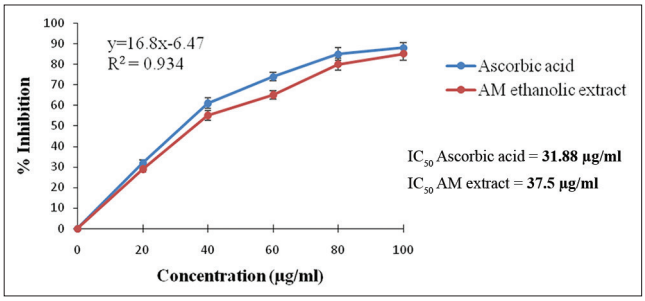

Fig. 3: Nitric oxide radical scavenging activity

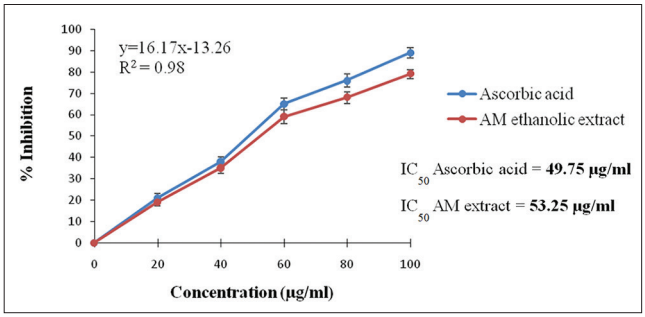

Fig. 4: Superoxide radical scavenging activity

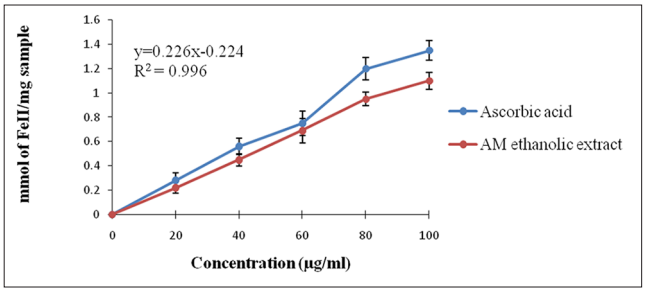

Fig. 5: Ferric reducing antioxidant power assay

Table 2: Phytochemicals in the ethanolic extract of whole plant of *Azolla microphylla*

RT	Compounds identification (tentative)	MF	Area %
16.92	1,3-di-iso-propylnaphthalene	C16H20	0.3
17.87	3-ethyldibenzothiophene	C14H12S	0.52
19.38	Tetradecanoic acid, 12-methyl ester	C16H32O2	0.51
20.27	Neophytadiene	C20H38	1.64
21.12	2-[(Z)-9-octadecenyloxy] ethanol	C20H40O2	0.23
21.59	9-hexadecenoic acid, methyl ester	C17H32O2	1.04
22.02	Hexadecanoic acid, ethyl ester	C17H34O2	4.8
22.75	3-cyano-12-isopropoxy-6,11-methanocyclodeca[g] imidazo[5,1-c](1,2,4) triazine	C18H15N5O	1.15
23.98	cis-13-octadecenoic acid, methyl ester	C19H36O2	1.05
24.88	Hexadecanoic acid, 2,3-dihydroxypropyl ester	C19H38O4	0.2
26.07	9-Octadecenoic acid	C18H34O2	15.04
28.75	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	C35H68O5	0.5
30.41	(1R)-2-(1S)-1-[2-(Methoxymethoxy) phenyl] ethyl} amino) oxy]-1-phenylethanol	C18H23NO4	0.69
31.67	3-[(tert-Butyldimethylsilyl) oxy]-1,4,4a, 9a-tetrahydro-1-phenyl-9H-xanthen-9-one	C25H30O3Si	0.76
32.89	Cholest-2-en-1-ol	C27H46O	0.39
33.33	Oleic acid, eicosyl ester	C38H74O2	0.34
34.94	(2R/S,4S/R,6R/S)-4-Hydroxy-2-tridecyl-1,7-dioxadispiro[5.1.5.2]pentadeca-9,12-dien-11-one	C26H42O4	2.04
35.82	3á-(Peroxymethyl)-5-vinyl-A, B-bisnor-5á-cholestane	C28H48O2	0.34
38.13	2,3,4,5,2',6'-Hexamethoxy-4',5'-methylenedioxychalcone	C22H24O9	55.47
39.28	Cucurbitacin B, dihydro-	C32H48O8	0.15
39.83	Chol-8-en-24-al, 3-hydroxy-4,4,14-trimethyl-	C27H44O2	0.17

RT: Retention time, MF: Molecular formula

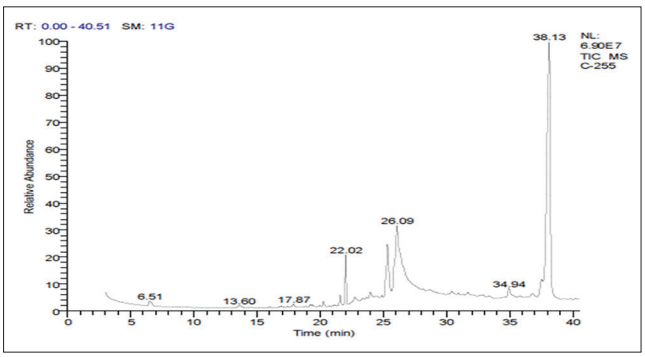

Fig. 6: Gas chromatography-mass spectrometry of phytochemicals present in ethanolic extract of *Azolla microphylla*

profound medicinal properties such as astringent, anti-inflammatory, and anti-microbial. The variation observed in the concentrations of analyzed phytochemicals with that of the previous findings, may be primarily due to various environmental factors such as climate, altitude, water quality, soil composition and intensity of sunlight exposure. Small quantities of alkaloids detected in *Azolla* species are in concordance with a previous report [26]. Adaptation to diverse habitats has indulged the pteridophytes to elicit remarkable genetic traits. Many of the taxa adapted to cold, desiccation and rains have been very peculiar to synthesize primary and secondary metabolites with unique characteristics, and they are called as "Evolved pteridophytes." In the present sample, such physiochemical variations observed in the evolved pteridophytes might be responsible for the synthesis of alkaloids and saponins in minute quantities. The detection of lower amounts of saponins in ferns is in harmony with the reports of Gracelin *et al.* [27].

The scope for the identification of natural antioxidants is ever increasing; owing to the toxic effects of synthetic antioxidants [28]. The current phytochemical study indicates an appreciable quantity of secondary metabolites and it may be scientifically convincing to expect the plant extract to elicit good antioxidant property. Hence, a set of different *in vitro* antioxidant assays were employed to cover most significant mechanisms by which different antioxidants reacts to inhibit the generation of free radicals.

The results of the DPPH free radical scavenging assay indicate that the ethanolic extract of AM exhibited strong scavenging activity on the DPPH free radical, which was increasing concomitantly with increasing concentrations of the plant extract. The IC_{50} value of L-ascorbic acid standard indicates the higher hydrogen donating capacity when compared to that of the extract. However, the IC_{50} value of the ethanolic extract of AM is substantial to be used as better scavenger of free radicals. These results are in concordance with the previous study [29].

The reduction of ABTS radical cation has been extensively used to investigate the antioxidant effect of the plant extracts [30]. A similar trend like that of DPPH results was observed in the IC_{50} values of the plant extract and standard. Interestingly, the presence of some unknown chemical compounds in the extract exhibited increased inhibition of ABTS radical formation, which is demonstrated by the lowered IC_{50} values.

The concentration of NO radical peaks during an infection or inflammation as it is an important chemical mediator generated for the regulation of various physiological processes. NO reacts with oxygen to form peroxynitrate anions that create adverse cellular damages such as DNA cleavage and membrane destabilization. [31]. In the present study, AM exhibited excellent scavenging activity against NO radicals (IC_{50} = 37.5 µg/ml) when compared to that of the other tested free radicals. In this assay, there observed no perceptible difference between the IC_{50} values of plant extract and standard ascorbic acid.

Physiologically, SO radical is considered as an important source for the formation of reactive oxygen species. Though it is considered as the weak oxidant, its relative products such as the hydroxyl radical and singlet oxygen are more powerful and harmful enough to create an oxidative stress [32]. The ethanolic extract of AM scavenged SO radical with an IC_{50} of 53.25 µg/ml while ascorbic acid being 49.75 µg/ml. This suggests that the ability to scavenge SO radical is nearly same for the plant extract and standard ascorbic acid under the tested conditions.

FRAP assay determines the total reducing power of the plant extract. In the presence of reducing agents, the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions was augmented, which is observed with a significant increase in the intensity of bluish-green color inside the reaction tubes [33]. Precisely, the increasing absorbance indicates the higher

reducing potential of the plant extract. In the present study, the reducing power of the AM extract as well as the standard ascorbic acid increased progressively in a dose-dependent manner.

The GC-MS analysis established the presence of compounds that are mainly derivatives of fatty acid, terpenoid, steroid, coumarin, and flavonoid. These compounds have been reported to possess various biological and pharmacological activities [34]. The highest area percentage value was observed for 2,3,4,5,2',6'-hexamethoxy-4',5'-methylenedioxychalcone. Chalcones are naturally occurring phytochemical found in various plant species like *Angelica, Glycyrrhiza, Humulus* and *Scutellaria* that are extensively used as traditional folk remedies [35]. In terms of area percentage the next major compounds detected were 9-Octadecenoic acid and Hexadecanoic acid ethyl ester of fatty acids. However, some minor quantities of other fatty acids such as cis-13-octadecenoic acid, methyl ester, 9-hexadecenoic acid methyl ester, tetradecanoic acid-12-methyl ester, oleic acid eicosyl ester and hexadecanoic acid-2,3-dihydroxypropyl ester were also observed. In general, the consumption of unsaturated fatty acids is implicated with serum cholesterol lowering effect, reduced risk of myocardial infarction and also aids in cancer prevention [36]. In addition, they possess potent biological properties such as nematicide, anti-arthritis, antioxidant, hepatoprotective and anti-microbial potential [37]. The results of Heilmann *et al.* suggest the antiprotozoal and cytotoxic activities of 1,7-dioxadispiro[5.1.5.2]pentadeca-9,12-dien-11-one derivatives isolated from *Amomum aculeatum* [38]. Interestingly, a similar derivative named (2R/S,4S/R,6R/S)-4-Hydroxy-2-tridecyl-1,7-dioxadispiro[5.1.5.2]pentadeca-9,12-dien-11-one detected in the present study might be capable of displaying similar properties. Neophytadiene is a terpenoid compound initially isolated from red alga, *Centroceras clavulatum*, which was later detected in plant species such as *Eupatorium odoratum.* It is known for its antifungal, antipyretic, analgesic, and anti-inflammatory potential [39]. The presence of such bioactive molecules in AM reinstates the pharmacological potential of this aquatic plant. Triazine derivatives are commonly known for its anti-mutagenic properties. Cucurbitacin was yet another interesting compound detected in ethanolic fraction of AM. They are oxygenated tetracyclic triterpenoid compounds, which are predominantly present in plants belonging to Cucurbitaceae. They exhibit various biological activities including anti-inflammatory, anti-microbial, and anti-tumor [40]. A negligible amount of compounds like 1,3-di-iso-propylnaphthalene and 3-Ethyldibenzothiophene were also detected, which is attributable to the presence of environmental contaminants. Aforementioned properties of bioactive molecules are very well studied in other plant species.

CONCLUSION

From the above research, it can be concluded that AM has immense antioxidant potential to be used in the areas of pharmacology and as a prospective source of valuable drugs. This may be due to the presence of significant quantities of phytochemicals such as tannins, phenols, flavonoids, saponins and alkaloids. This extensive research on compounds present in the ethanolic extract of AM makes this study novel and useful, thereby, justifying the incorporation of AM for treating various diseases. Nevertheless, the detected compounds are only tentative and it is recommended to isolate and characterize the individual phytochemical constituent and subjecting it to biological/toxicological assays, to obtain more harmonious results.

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