Original Article

GC-MS Analysis of Bioactive Compounds and Host-toxicity Studies of *Azolla caroliniana* Symbiotic with the Cyanobacterium *Anabaena azollae*

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ABSTRACT

Objective: The present research work was conducted to do the gas chromatography and mass spectrometry (GC-MS) analysis of the water fern, *Azolla caroliniana* symbiotic with the cyanobacterium *Anabaena azollae*, along with *in vitro* host toxicity testing to check its toxicity level. **Materials and methods:** The GC-MS analysis of the methanol extract of *A. caroliniana* was carried out using a GC-MS instrument and *in vitro* host toxicity testing of *A. caroliniana* extract was carried out with cultured lymphocytes from human umbilical cord blood. **Results:** It was evident that only one phytochemical, 3-o-methyl-d-glucose with retention time 16.581 min and a peak area of 91.89% as the major phytochemical was present in methanolic extract of the fern, while the rest 7 of the total 8 chemicals namely (with peak area values), '2-butanone, 3-methoxy-3-methyl' (0.865%), '2,2-dimethyl propionic acid, cyclopentyl ester' (0.670%), 'butane, 1-bromo-2-methyl' (0.398%), '2-hexen-1-ol,2-ethyl' (0.269%), '5-hydroxy-2,2-dimethyl hexan-3-one' (0.212%), 'phthalic acid, ethyl pentyl ester' (0.21%), 'pentanoic acid, 2-methyl' (0.182%), were present in minor quantities. Host toxicity testing was done with *in vitro* cultured lymphocytes from human umbilical cord blood. The LC_{25} values were 870.96 and 691.83 mg/L with the trypan blue and acridine orange/ethidium bromide staining, respectively by methanolic extract of *A. caroliniana* in cellular toxicity experiments, against the minimum inhibitory concentration values of 300 mg/L extract. During the assessment of nuclear toxicity, no comet was found in the cells grown with 0 to 1000 mg/L of the extract. **Conclusion:** The extract had neither cellular nor nuclear toxicity to cultured human lymphocytes.

Key words: *Azolla caroliniana*, Fern, Gc-Ms Analysis, 3-O-Methyl-D-Glucose, Host Toxicity Testing, *In Vitro* Cultured Lymphocytes.

INTRODUCTION

One among 7 species of *Azolla* (Salviniaceae), *A. caroliniana* Willdenow, the 'mosquito/water fern', is a small fast-growing free-floating hydrophyte of idle tropical/temperate fresh waters, worldwide.[1] *Azolla* sp. is traditionally used as a bio-fertilizer in Asian rice farming by growing and mixing into rice-soil during puddling, as well as an animal feed mainly.[2,3] Because of its symbiosis with the N_2-fixing cyanobacterium, *Anabaena azollae*, it adds nitrogenous compounds to the growing environment and helps soil-conditioning.[1-3]

The wide use of *Azolla* sp. as cattle and poultry feed and limited use as human food, particularly seen in China is known.[4] Its antioxidant capabilities were assessed with *A. caroliniana*, with several biochemical methods, with a record of its antibacterial capabilities in parallel, with 7 human pathogenic multidrug resistant (MDR) bacteria isolated from clinical samples. Surprisingly, antioxidant and antibacterial capabilities of the species were blithely confirmed, since both activities are of rare occurrence in a plant. Preliminary phytochemical analyses

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of fern-extracts with solvents, chloroform, ethanol, methanol and n-hexane for the presence of alkaloids, anthraquinones, carbohydrates, flavonoids, glycosides, phenols, resin, saponins, steroids, tannins and terpenoids were recorded.[5] Intuitively, there had been a logistic interest in detailing the phytochemical contents of biologically active extract of *A. caroliniana,* due to its dual beneficial medicinal role.

However, the first attempt on the phytochemical composition of the Indian species, *A. filiculoides* described the presence of C-glycosyl flavones.[6] A comparative study of *A. pinnata* extracted with acetone, benzene, chloroform, water, ethanol and petroleum ether reported presence of phenols, tannins, flavonoids and saponins.[7] A preliminary examination of the phytochemical profile of *A. microphylla* too indicated the presence of sterols, tannins, flavonoids and glycosides, moderately.[8] Moreover, two flavonoids, rutin and quercetin having antioxidant activity were identified from another Indian species, *A. microphylla*.[9] In the previous work on *A. caroliniana* it was found that methanol extract of plant had considerable amounts of phenolic compounds justifying its antioxidant capability; additionally, methanol, ethanol and chloroform extracts of *A. caroliniana* were recorded the presence of flavonoids, phenols, saponins and tannins.[5] Methanolic extract of *A. microphylla,* was used for assessing antibacterial activity *in vitro* against with bacterium, *Xanthomonas oryzae* pathogenic on rice and bioactive compounds as a mixture of eicosenes and heptadecanes were reported with a GC-MS analysis of the ethyl acetate fraction.[10]

This study on gas chromatography and mass spectrometry (GC-MS) analysis of methanolic extract of *A. microphylla* was the continuation of the previous study on the antibacterial and antioxidant properties of the fern,[5] for assessing its bioactive compounds in detail. In addition, host toxicity testing of the fern extract of with *in vitro* cultured lymphocytes from human umbilical cord blood was pursued, similar to the recently surging 'predictive toxicology' work, described on a chemical of environmental concern,[11] since host toxicity testing is an essential corollary to such a scientific assertion of a non-conventional (but generally known as non-toxic) Indian plant species as a food-grade plant for man, in corroboration to the previous nutrition-level checking report[12] and organoleptic work.[5] Furthermore, testing of cellular and nuclear toxicity with a human cell line should be regarded as an attempt of revealing the consequence of exact mirror of untoward body events in any, from the use of *Azolla* as a food grade plant. Obviously, the use of whole animals is the most coveted method of host toxicity testing of any new chemical/food grade material, traditionally in pharmacology. Nevertheless, the later method is too under circumspection as the most dependable method, for the reason of the suppression of probable toxic events due to physiological homeostasis of live animals that might be of different in man, despite the fact that most conventional experimental animals are mammals. In the present study, the methanolic extract of *A. caroliniana* was analyzed by GC-MS study, and several bioactive compounds were identified. This work is anticipated that, *A. carolinian* could be recommended as supplementary food after conformation of its detailed chemical contents and absence of any toxic effects holistically to host, verified with a human cell culture line *in vitro*.

MATERIALS AND METHODS

Collection and extract preparation

Azolla caroliniana was collected from a local animal farm, where it was grown in a well illuminated cement tank. Fern-fronds were harvested and were shade dried. Methanolic extract was prepared by the cold extraction method from the dried mass, using a vacuum rotary evaporator for a semisolid mass as described.[5] The extract was further stored in small vials in 10% dimethyl sulfoxide (DMSO), for a concentration of 100 mg/mL.

GC-MS analysis

The GC-MS analysis of the methanol extract was carried out using an instrument equipped with a VF-5 ms fused-*silica* capillary column of 30 m length, 0.25 mm diameter, and 0.25 mm film thickness. An electron ionization system with ionization energy of 70 eV was used as the detector. Helium gas (99.99%) was used as the carrier gas at the constant flow-rate of 1.51 mL/min. The temperatures of the injector and mass transfer line were set at 200ºC and 240ºC, respectively. The oven temperature was programmed from 70ºC to 220ºC at 10ºC/min, held constant for 1 min and finally increased to 300ºC at 10ºC/min. Aliquots of 2 mL of the diluted samples were manually injected in the split-less mode with a split ratio of 1:40 and with a mass scan range of 50e600 AMU. The total running time of the GC-MS analysis was 60 min.[13]

Identification of compounds

The phytochemical components of the biologically active methanolic extract was identified by comparing their fragmentation pattern of mass spectra and retention indices with those stored in the following databases: NIST08.LIB (Stein SE National Institute of Standards and Technology, Mass Spectral Database and Software. Version 3.02, NIST, Gaithersburg, MD, USA, 1990) and

WILEY8.LIB[14]; and comparisons with other published work of mass spectra were done.

Host toxicity study with human lymphocytes

Isolation of lymphocytes

Human umbilical cord blood (UCB) was collected in a sterile 15 or 50 mL size falcon tube (Tarson, Kolkata), with an aliquot of 100 or 250 µL 1,000 IU heparin (HiMedia, Mumbai), immediately after the delivery of an infant. And the blood sample (less than 15 to 50 mL) was stored at 4ºC till use; lymphocytes were isolated immediately or within at the best 24 h after the collection. For the isolation of lymphocytes, the collected UCB sample was diluted with an equal volume of phosphate buffered saline (PBS) solution. Further, the mixture was loaded carefully into a centrifuge tube for over-layering with lymphocyte separating medium (LSM, HiMedia), which was one-third the total volume of the mixture. The mixture was centrifuged at 1,800 rpm for 25 min at 22-24ºC, as a result four heavy to light layers, red blood cells (RBC), LSM, buffy coat and plasma were seen. The buffy coat layer with mononuclear cells was taken out carefully from the tube. To the separated cells of the buffy coat layer, after the addition of another aliquot of PBS for the 1:1 ratio, re-centrifugation at 2,000 rpm for 5 min was done. The pellet of lymphocytes was taken for culturing and cell counts were done using a haemocytometer.[11]

Culture of lymphocyte

After separation, UCB-derived lymphocytes were diluted to the density of $1x10^6$ cells/mL with a required volume of Dulbecco's modified Eagle's medium (DMEM-low-glucose, HiMedia), and were loaded into a 6-well culture plate (Tarson), which contained 15% fetal bovine serum (FBS, Sigma), 1% penicillin-streptomycin and 1% sodium pyruvate, along with graded concentrations of *A. caroliniana* extract for growth. The volume of 2 mL in total was maintained in each well of the culture plate with the extract at different concentrations. The cells were incubated with different concentrations of *A. caroliniana* extract (0, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mg/L) in an incubator at 37 ºC in 5% atmospheric CO_2 concentration for 24 h for growth *in vitro*.[11]

Monitoring cellular toxicity

The viability of lymphocytes grown in the presence of the extract was assessed using two staining procedures, with trypan blue (TB) and acridine orange/ethidium bromide (AO/EB), using phase contrast microscope (Magnus) and a fluorescent, respectively.

TB staining

TB solution was prepared in PBS at the concentration of 0.4%. For the study of cell viability, to the *in vitro* grown mass of lymphocytes, TB solution was added at the 1:1 ratio. Then the mixture was kept in an incubator for 2 min at 37ºC; cells in triplicates were observed under a phase-contrast microscope (Magnus) at 400x magnification. The live cells remained unstained whereas; the nuclei of dead cells were blue in appearance, as TB is a membrane permeable dye that enters dead cells and stains the nuclei blue.

AO/EB staining

The AO/EB solution was prepared in PBS at the concentration of 100 µg/mL. To the lymphocytes grown at different concentrations of *A. caroliniana* extract, the AO/EB solution was added at 4 µL to 96 µL lymphocytes. When observed under the fluorescent microscope (Magnus at 400X), green colour indicated live cells, whereas cells with orange and red colour were apoptotic and necrotic cells, respectively. Lethal percent values were transformed to probit values (Finney's method), which were plotted against corresponding $\log_{10}$ values of *A. caroliniana* extract concentrations, as exemplified before.[11] Probits of observed lethality percentage values are from statistical tables of probit transformation, as described recently.[11]

Comet assay

Single cell gel electrophoresis was carried out to study nuclear/ DNA damage of the lymphocytes grown with graded concentrations of *A. caroliniana* extract. Cultured cells were harvested and used in the alkaline comet assay technique. After coating slides with 1% agarose were air dried. Cultured lymphocytes individually from each *A. caroliniana* extract concentration were centrifuged and pellets were washed with PBS; and the washed cells were mixed with three times the cell volume with the low melting point agarose (LMPA) 1% in sol state. The mixture of cells and LMPA sol was placed over the agarose coated slide that was kept at 4ºC for 10 min for it to get dried. The slides were further treated with 1% Triton X 100, 10% DMSO, individually, and were placed in the lysing solution of the mixture of 100 mM Na_2EDTA, 10 mM Tris, 2.5 mM NaCl (pH, 10), at 4ºC for 1 h. The slides were subsequently removed and placed in the electrophoretic buffer consisting of 1 mM Na_2EDTA and 300 mM NaOH (pH 13) for 30 min. The slides were transferred to a horizontal gel electrophoretic chamber with electrophoretic buffer. Electrophoresis was carried out at 1.0 V/cm for 30 min. After the electrophoresis, the slides were placed in the

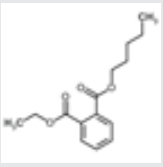
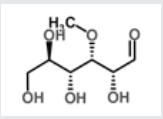
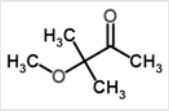
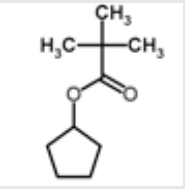
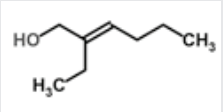
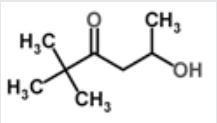
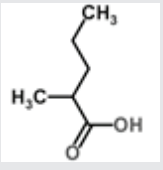
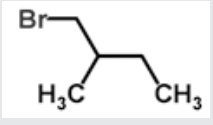
neutralizing solution (0.4 M Tris HCl, pH 7.5) for 5 min. The slides were stained with an aliquot of 40 µL of 10 µg/mL ethidium bromide solution.[15] The slides were observed under the fluorescence microscope at 400 X.[11]

RESULTS

GC-MS analysis of *A. caroliniana*

From the GC-MS analysis of the methanolic extract, the presence of 8 phytocompounds was evident in the chromatogram (Figure 1). Chemical profiles of the identified compounds were ascertained together with their retention time (RT), percentage of peak area, molecular formula, molecular weight and chemical structure (Table 1). Studies on the phytochemicals of *A. caroliniana* by the GC-MS analysis clearly showed the presence of 8 compounds, as follows with RT as min in the order, 15.551, 16.581, 18.022, 18.212, 18.392, 18.582, 19.002 and 20.762 min, respectively: A, phthalic acid, ethyl pentyl ester (0.21%); B, 3-o-methyl-d-glucose (OMG) (91.89%); C, 2-butanone, 3-methoxy-3-methyl (0.865%); D, 2,2-dimethylpropionic acid, cyclopentyl ester (0.670%); E, 2-Hexen-1-ol, 2-ethyl (0.269%);

Table 1: Phyto-compounds identified from methanol extracts of *A. caroliniana*

Peak	RT	Area	Area %	Name of compound	MF	MW	Molecular structure
A	15.551	58,207.1	0.21	Phthalic acid, ethyl pentyl ester	$C_{15}H_{20}O_4$	264	
B	16.581	25,191,662	91.89	3-O-Methyl-d-glucose	$C_7H_{14}O_6$	194	
C	18.022	237,223.1	0.865	2-Butanone, 3-methoxy-3-methyl	$C_6H_{12}O_2$	116	
D	18.212	183,612.5	0.670	2,2-Dimethyl propionic acid, cyclopentyl ester	$C_{10}H_{18}O_2$	170	
E	18.392	73,650.7	0.269	2-Hexen-1-ol, 2-ethyl	$C_8H_{16}O$	128	
F	18.582	58,129.3	0.212	5-Hydroxy-2,2-dimethyl hexan-3-one	$C_8H_{16}O_2$	144	
G	19.002	49,967.7	0.182	Pentanoic acid, 2-methyl	$C_6H_{12}O_6$	116	
H	20.762	109,082.3	0.398	Butane, 1-bromo-2-methyl	$C_5H_{11}Br$	150	

Remark: RT = retention time (min), MF = molecular formula, MW = molecular weight.

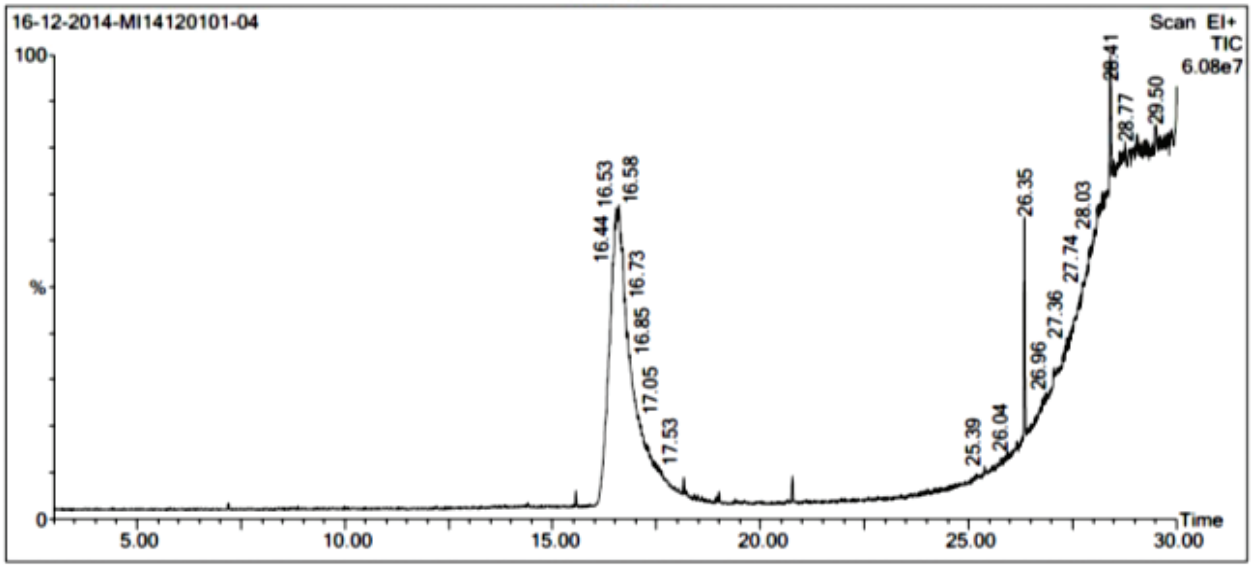


Figure 1: Gas chromatography-mass spectrometry chromatogram of methanolic extract of *A. caroliniana*.

Table 2: Lethality values during *A. caroliniana* toxicity to cord blood lymphocytes growing in DMEM, assessed by two methods, AO/EB staining as mean with ± standard deviations along with probits of lethal values

Concentrations of *A. caroliniana* extract (mg/l)	Log_{10} Concentrations	Lethal percent value with AO/EB staining	Probit values with AO/EB staining	Lethal percent value with TB staining	Probit values with TB staining
0	0	0	0	0	0
100	2.0	0	0	0	0
200	2.301	0	0	0	0
300	2.477	4.6±2.08	3.32	5.0±2.64	3.35
400	2.602	10.6±1.52	3.75	8.6±2.51	3.63
500	2.698	10.6±1.52	3.75	10.6±1.52	3.75
600	2.778	21.0±2.64	4.20	20.0±2.64	4.16
700	2.845	24.0±1.00	4.30	10.6±1.52	3.75
800	2.903	21.0±2.64	4.20	23.6±2.08	4.28
900	2.954	30.6±3.51	4.50	24.0±1.00	4.29
1000	3.0	33.6±3.57	4.57	29.6±2.08	4.46

Remark: AO/EB = Acridine orange/ethidium bromide, TB = Trypan blue, DMEM = Dulbecco's modified Eagle's medium

F, 5-hydroxy-2,2-dimethylhexan-3-one (0.212%); G, pentanoic acid, 2-methyl (0.182%); H, butane, 1-bromo-2-methyl (0.398%) (Table 1, Figure 2 A to 2H). The OMG (Figure 2B) has synonyms, 2,4,5,6-tetrahydroxy-3-methoxy-hexanal and methyl glucose (https://www.wikigenes.org/e/chem/e/298225.html). However, the rest 7 chemicals, marked as, A, C. D, E, F, G and H (Figure 2) were in minor quantities.

Assessments of *A. caroliniana* extract host toxicity

Lethal percent values recorded from data sets of cellular toxicity tests with AO/EB and TB staining methods of cultured lymphocytes were used along with log_{10} concentrations of the *A. caroliniana* extract to construct respective probit plots, which were used for extrapolation to compute individual LC_{25} values.

TB staining indicated that after growing lymphocytes with graded concentrations of *A. caroliniana* extract at 0 to 1000 mg/L, the minimum inhibitory concentration (MIC) value after TB staining was recorded as 300 mg/L extract. From the probit plot, it was ascertained that for the value of LC_{25} (probit value 4.3255), the corresponding log_{10} concentration value was 2.84. Antilog value of

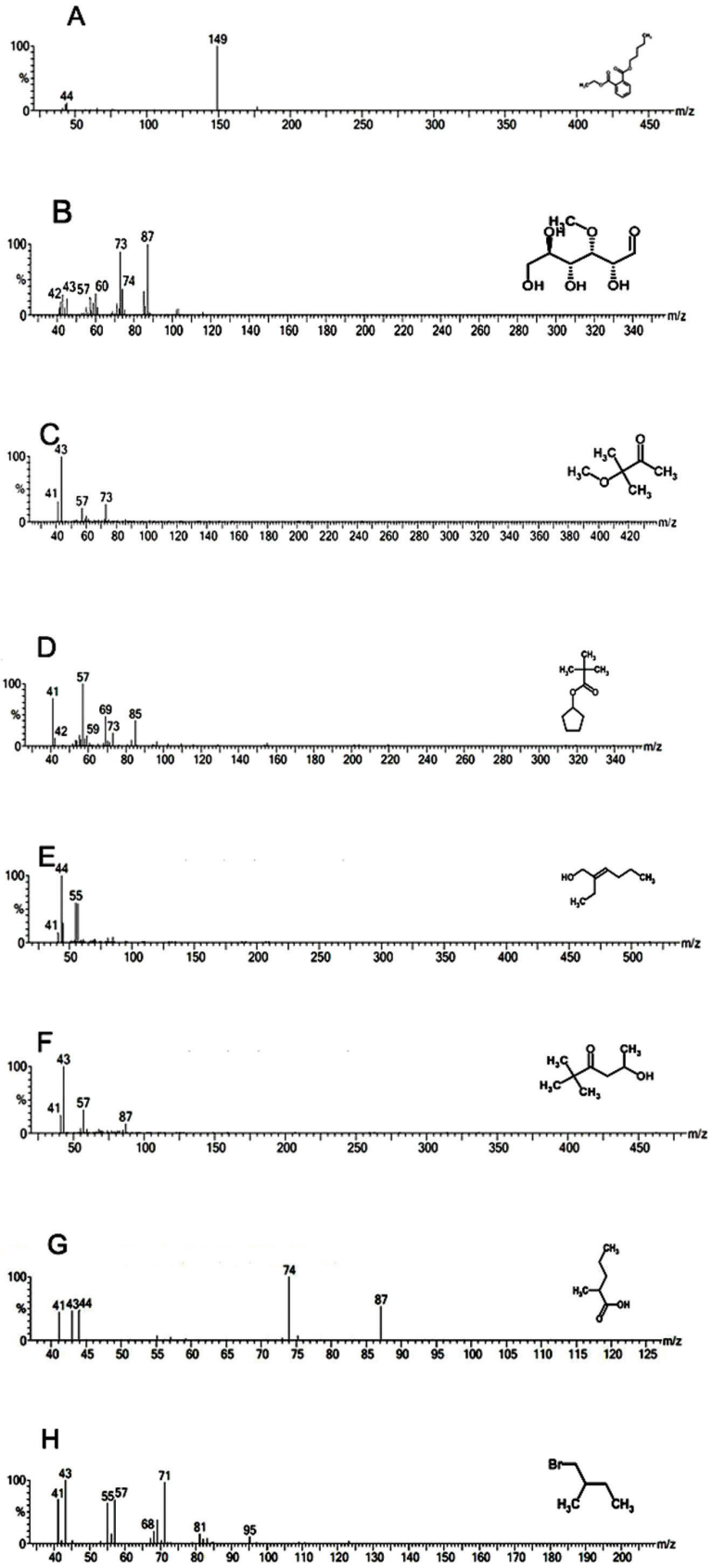


Figure 2: Mass spectra of 8 compounds, identified from methanol extract of *A. caroliniana* with structures as individual inserts, (A) phthalic acid, ethyl pentyl ester, (B) 3-o-methyl-d-glucose, (C) 2-butanone, 3-methoxy-3-methyl (D) 2,2-dimethylpropionic acid, cyclopentyl ester, (E) 2-hexen-1-ol, 2-ethyl, (F) 5-hydroxy-2,2-dimethylhexan-3-one, (G) pentanoic acid, 2-methyl, (H) butane, 1-bromo-2-methyl.

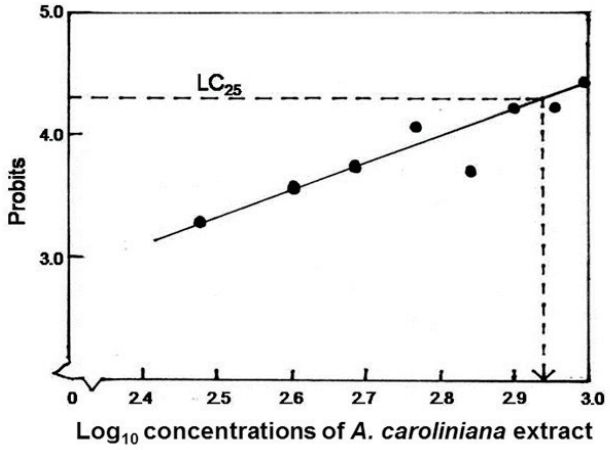


Figure 3: A plot of probits of lethal percent values against log_{10} concentrations of methanol extract of *A. caroliniana* in the cell toxicity during growth of lymphocytes with the graded levels of the extract assessed by TB staining method; the plot was used for the determination of extract concentrations for LC_{25} level of toxicity.

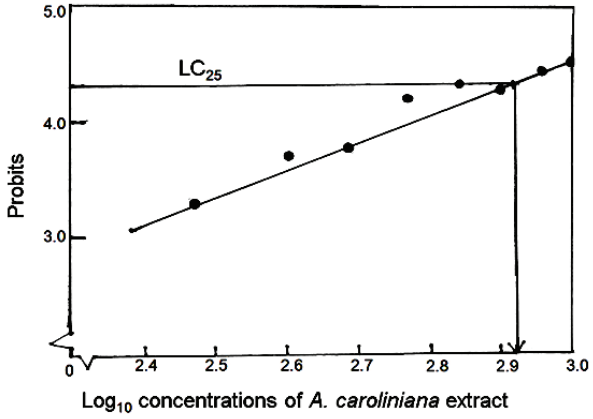


Figure 4: A plot of probits of lethal percent values against log_{10} concentrations of methanol extract of *A. caroliniana* in the cell toxicity during growth of lymphocytes with the graded levels of the extract assessed by AO/EB staining method; the plot was used for the determination of extract concentrations for LC_{25} level of toxicity.

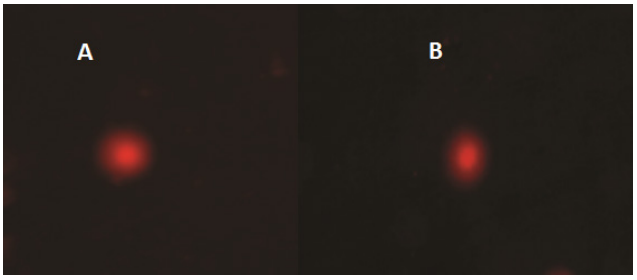


Figure 5: Comets to assess nuclear toxicity by the fern extract, (A) Control cells, (B) Cells had no comets when grown with 1000 mg/l of *A. caroliniana* extract.

this log_{10} concentration value was the LC_{25} value, 870.96 mg/L *A. caroliniana* extract (Figure 3).

Growth of lymphocytes with varying levels of *A. caroliniana* extract for 24 h showed the MIC at 300 mg/L extract. Probit values after AO/EB staining (Table 2) were used in the ordinate and log_{10} values of *A. caroliniana* extract concentrations in the abscissa for the construction of the probit plot, from which it was ascertained that the LC_{25} value was 691.83 mg/L extract in this method (Figure 4).

Single cell gel electrophoresis was carried out to study DNA damage of the cells grown with different concentrations of *A. caroliniana* extract. It was observed that, comet was not found in the cells cultured with 0 to 1000 mg/L extract (Figure 5).

The LC_{25} value was 870.96 mg/L *A. caroliniana* methanolic extract in the TB staining method, while the LC_{25} value was 691.83 mg/L extract in the AO/EB staining method. But experimentally, the MIC value was 300 mg/L extract. LC_{25} is the concentration where 25% cell death occurs. Herein, MIC was far less than any of the two LC_{25} values in staining methods obtained by computation for cell death. Thus, the extract had no cellular toxicity, for the reason that LC_{25} value is higher than the MIC value. Since, no comet was found due to the extract, the nuclear toxicity was not too induced by it.

DISCUSSION

In the previous study, the solvent extracts of *A. caroliniana* were recorded having antibacterial activity against seven MDR pathogenic bacteria, among which most Gram-negative and Gram-positive bacteria were highly resistant to 17 currently used antibiotics.[5] The methanol extract of *A. caroliniana* was recorded the best antibacterial activity against *S. aureus in vitro*, controlling the bacterial lawn at a MIC value of the methanolic extract, 1.89 mg/mL; the highest concentration required for the total control any of 7 MDR bacterium was 21.67 mg/mL methanolic extract as the minimum bactericidal concentration value.[5] Thus, both antioxidant and antibacterial efficacies of the fern were elucidates. This work on chemical profiling of the fern as well as, toxicity analysis with umbilical cord blood lymphocytes adds to the confidence that this could be further cultivated aesthetically for use as supplementary food, and the Chinese uses as food is justified. There were 6 other minor phytochemicals. Synergistically, all phytochemicals should be contributing to the recorded antioxidant and antibacterial activity of *A. caroliniana*. Herein, it is recorded that even the value of 300 mg/mL methanolic extract of *A. caroliniana* is nontoxic to human lymphocyte cultures.

Thus it could be suggested for the use of *A. caroliniana* as CAM during treatment if infection along with main stream medicines, i.e., antibiotics and other antibacterials. Additionally, the ability of the fern to produce an array of secondary metabolites, like several higher plants[13-16] is considered important for the use against bacterial pathogens as a non-microbial antimicrobial agent against the array of bacterial self defense mechanisms, i.e., shenanigans in achieving resistance over applied antibacterials[17] since the symbiotic fern has a higher litany of chemicals that could never be won upon by any prokaryote, equipped with armamentaria of multi-resistance[18] The present finding of the absence of host-toxicity similar to the medicinal plants, *Woodfordia fruticosa*[13] and *Combretum albidum*[16] with human lymphocyte culture supported the use of the fern as complementary and alternative medicine (CAM), along with mainstream antibiotics. The concept of CAM of using phyto-drugs is now widely held, being promoted by World Health Organization[19], and in the future it would become deeply held for failure of main stream medicine for several grimmish ailments. In the developed world too, the use of phyto-drugs as CAM is the new emerging social paradigm.[19]

In the previous report, the presence of flavonoids in alcoholic extracts of the species was the probable causative agents for the recorded antioxidant activity,[5] as known with higher plants.[20]Furthermore, the presence of phenols in all 4 solvent extracts of *A. caroliniana* was reported,[5] and the phenolic acid is known to be responsible for the antimicrobial activity of higher and lower plants.[21]

OMG is a nontoxic, non-metabolizable glucose analogue, effective in reducing the toxicity of streptozotocin (causative natural chemical of cancer development in islets of langerhans of pancreas in human); 3-o-methyl-d-glucose or OMG has been reported to possess anti-tumor properties.[22] Additionally, OMG has several biologic actions, and the most leading one is that this sugar is rapidly taken up by insulinoma cells (benign tumour cells of pancreas, in patients with hypoglycemia) (https://www.wikigenes. org/e/chem/e/298225. html). As OMG is amply present in *A. caroliniana,* a vivid research with animal models for its possible use as CAM for people suffering from malfunction of secreted insulin, resulting in frequent hypoglycemia, who often have insulinoma cells. Such a high percentage, 91.89% of OMG in *A. caroliniana* qualifies the plant aptly as a potent antioxidant too. Eventually, further work with chemicals of the species, if done with finesse could open up a field of locating/aiding to anticancer drugs.

Likewise, another compound located in *A. caroliniana*, 'phthalic acid, ethyl pentyl ester' is a derivative of phthalic acid**,** an aromatic compound, reported being too isolated from the medicinal plant, *Leea indica* (Burm. F) Merr flowers with esters of phthalic acid at 95.6% had remarkable antibacterial and antifungal activities.[23] Thus, the reported antibacterial efficacy of *A. caroliniana,* even against MDR pathogenic bacteria[5] could be related to the presence of this chemical. However, the remaining 6 phytochemicals seen in this GC-MS analysis could contribute to antioxidant/antimicrobial potencies of the fern. Despite, the bioactivities of these compounds isolated from other plants are not reported yet, as searched systematically in databases, all phytochemicals together have a natural synergistic action, as reported for an Indian herbal formulation.[24] To sum up, it could be stated that *Azolla* sp. would more suitable as a natural source of antioxidants than as a source of antimicrobials, for the presence of OMG, apart from several other phytochemicals. When this could be verified with *in vivo* experiment models, a prominent business tycoon could be initiated with *Azolla* sp.

CONCLUSION

It was evident in the present study that only one phytochemical, 3-o-methyl-d-glucose with RT 16.581 min and a peak area of 91.89% was present largely in *A. carolinianaa* and it is a glucose analogue along with its well proven antioxidant activity, it could be taken as a food grade palnt. Additionally, the second most prevalent chemical, 'phthalic acid, ethyl pentyl ester' has remarkable antibacterial properties. Thus the plant extract has double beneficial effects antioxidant and antibacterial activates. There were 6 other minor phytochemicals, which synergistically contribute to the major activity of *A. caroliniana.* Its methanolic extract had neither cellular nor nuclear toxicity with cultured human lymphocytes. If it could be grown aesthetically, *Azolla* sp. could be taken as safe, food grade plant.

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CONFLICT OF INTEREST

The authors have no conflict interest.

ABBREVIATIONS USED

AO/EB: Acridine orange/ethidium bromide; **CAM:** Complementary and alternative medicine; **DMEM:** Dulbecco's modified Eagle's medium; **DMSO:** Dimethyl sulfoxide; **FBS:** Fetal bovine serum; **GC-MS:** Gas chromatography and mass spectrometry; **LC:** Lethal concentration; **LMPA:** Low melting point agarose; **LSM:** Lymphocyte separating medium; **MDR:** Multidrug resistant; **MIC:** Minimum inhibitory concentration; **OMG:** 3-o-methyl-d-glucose; **RT:** Retention time; **PBS:** Phosphate buffered saline; **RBC:** Red blood cells; **TB:** Trypan blue; **UCB:** Umbilical cord blood.

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SUMMARY

- *Azolla caroliniana*, the water fern, symbiotic with the N2-fixing cyanobacterium, *Anabaena azollae* is traditionally used as a bio-fertilizer in Asian rice farming, and an animal fodder as well as, occasionally as human food.
- The GC-MS analysis of the methanol extract of *A. caroliniana* was carried and *in vitro* host-toxicity testing was carried out with cultured lymphocytes from human umbilical cord blood.
- From the GC-MS analysis total 8 phytochemicals were identified, and 3-O-methyl-d-glucose was the major phytochemical with a peak area of 91.89%.
- During host toxicity testing, methanol extract had neither cellular nor nuclear toxicity to cultured human lymphocytes.
- If it could be grown aesthetically, *A. caroliniana* could be taken as safe, food grade plant.

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