Chemical and Biological Technologies in Agriculture

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Investigation of important biochemical compounds from selected freshwater macroalgae and their role in agriculture

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Abstract

Background: Freshwater macroalgae possess a number of important secondary metabolites. They are an unexplored source of medicinal compounds. In this study, three freshwater macroalgae—*Chara vulgaris*, *Cladophora glomerata* and *Spirogyra crassa*—were collected from the river Swat and the river Kabul in the Charsadda district of Khyber Pakhtunkhwa, Pakistan. To assess the role of freshwater macroalgae in agriculture, various experiments were performed on their extracts. Methanolic extract of the three macroalgae were first analyzed through gas chromatography–mass spectrometry (GC–MS) for the presence of important medicinal secondary metabolites. The methanol based macroalgae extracts were tested for antibacterial, insecticidal, cytotoxic and phytotoxic activities.

Results: Initially, the algae were dried, crushed and treated with methanol for the extraction of secondary metabolites. The GC–MS results contained several important long chain fatty acids and other related long-chain hydrocarbons, such as alkanes and alkenes. Several benzene derivatives were also detected during the course of the investigation. Several of these compounds have established roles in the treatment of human ailments and can be supplied to farm animals. For example, phenylephrine is a decongestant, dilates pupils, increases blood pressure and helps in relieving hemorrhoids. Hexahydropseudoionone has uses in perfumes and other cosmetics. Several essential oils were also detected in the methanolic extract of the three macroalgae that can be utilized in various industrial products. Bioassays showed that these algal extracts—especially the *Spirogyra* sp. extract—contain moderate to maximum bioactivity.

Conclusions: Macroalgae possess important secondary metabolites with medicinal properties. These secondary metabolites can be used as biopesticides, plant growth enhancers, and remedies for various diseases in farm animals and for the control of weeds. They can be further explored for isolation and purification of useful biochemical compounds.

Keywords: Essential oils, Macroalgae, Gas chromatography, Mass spectrometry, Fatty acids, Bioassays

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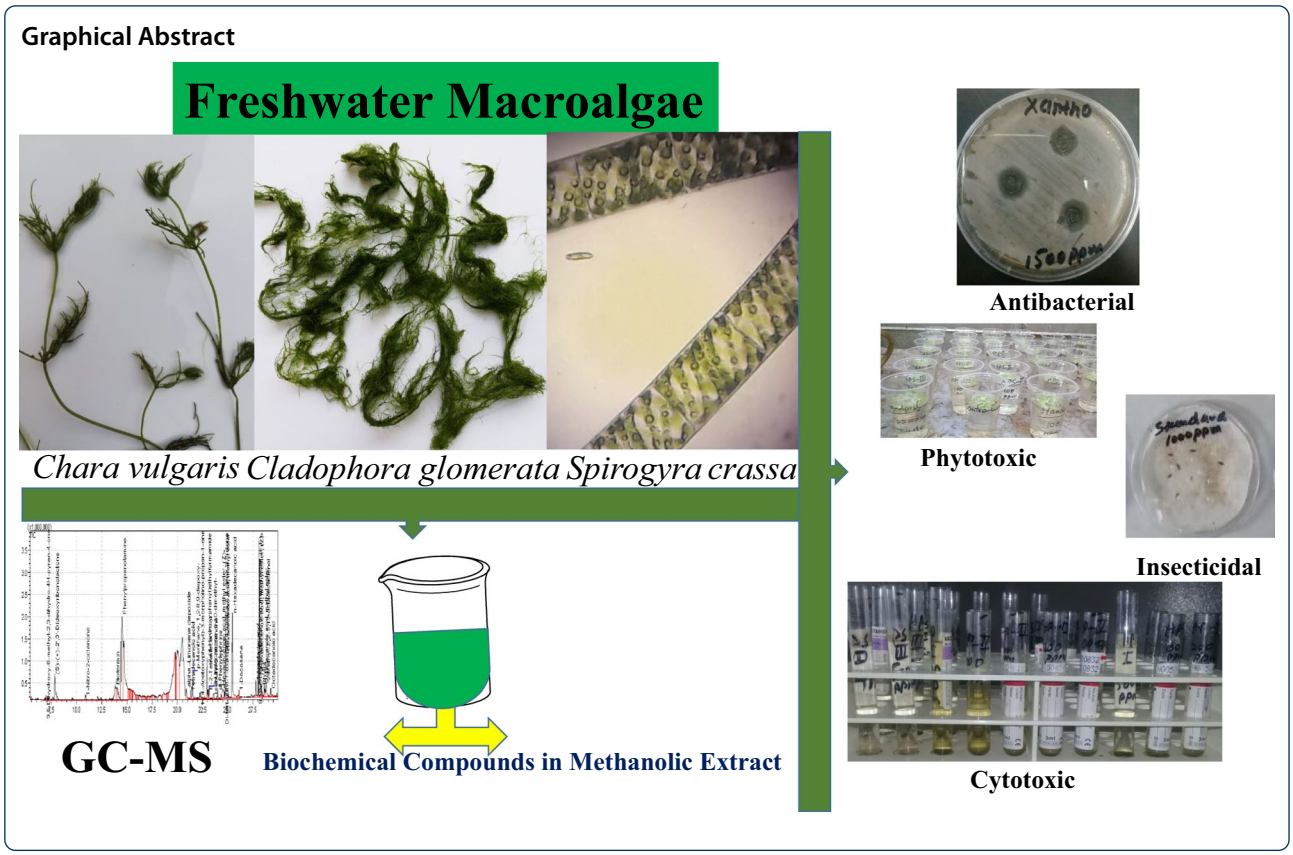

Introduction

Algae are a heterogeneous group of plant-like organisms that belongs to the kingdom Protista. They are comprised of the dominant photoautotrophs, from simple microscopic or macroscopic to multicellular plants, present in diverse aquatic habitats from freshwater bodies to vast marine water [1]. Actively growing algae convert the sun's energy into a diverse group of metabolites called bioactive compounds. These bioactive substances include carbohydrates, proteins, lipids, phenols, vitamins, free amino acids, enzymes and growth regulators [2]. Phycologists have investigated various forms of natural bioactive compounds present in marine algae from all over the world. A number of fatty acids (both saturated and unsaturated), terpenes, sterols and carbohydrates have been extracted from different algae. In contrast to comprehensive data available on marine algae, phytochemical information for freshwater macroalgae is incomplete [3]. Members of the Chlorophyceae class of green algae are a source of bioactive compounds with diverse chemical structures and biological activities, with an approximately 3.98 billion dollar market in 2018 [4]. Both marine and freshwater algae are a good source of pharmacologically active metabolites [5, 6]. The presence of bioactive secondary metabolites with antitumor activity [6], antibacterial [7], antioxidant [8], and anti-inflammatory [8] activities has been recognized in various macroalgae as they contain active biological phytochemicals, such as fatty acids, sterols, peptides, proteins, polysaccharides, heterocyclic carbons and terpenes [9]. *Cladophora*, for example, has more than 183 species and all have diverse phytochemicals with medicinal properties [10].

Algal products can be used for sustainable agriculture; to increase plant growth and soil nutrients, control soil toxicity, and as bio-fertilizers and bio-pesticides [11, 12]. Extracts from algal species including *Chlorella*, *Saragassum*, *Laminaria*, *Durvillaea*, *Ascophyllum*, and *Ecklonia* have biostimulant effects on strawberry and other agriculture crops. Algal extracts also contain secondary metabolites that have antifungal properties and have the ability to control various crop fungal diseases [13]. The extract of *Spirulina platensis* has the ability to increase the biomass of the reddish plant. Its extract contains bio-stimulants that increase plant growth [14]. Several cyanobacteria and algae have the capability to fix different forms of nitrogen and thus increase the fertility of the environment for plant growth. They can decrease the demand for nitrogen fertilizers by 25%. Several algae produce biocidal agents that include benzoic acid and majusculonic acid. These compounds are toxic to different

nematodes, fungi and other harmful species that attack plants. Algae perform 50% of the world's photosynthesis, therefore, playing a major role in carbon dioxide sequestration and oxygen production [15–17]. Due to fast growth and enormous biomass production, algae are favorable sources of bioenergy [18, 19]. *Ulva* extracts can help in germination and increases root size in *Arabidopsis*. *Ulva* extract contains hormones and biostimulants for plant growth, which help to increase biomass [20]. In the present work, we have investigated the chemical composition of methanolic extract from the freshwater macroalgae *Chara vulgaris*, *Cladophora glomerata*, and *Spirogyra crassa*, that prevail in the river Swat and Kabul. Using gas chromatography–mass spectrometry (GC–MS), this work analyzes the availability of different fatty acids (saturated and un-saturated) and other phytochemical components in essential oils of methanolic extract. Furthermore, the bioactivities of the methanolic extracts of these selected freshwater green macroalgae are examined, to explore their possible medicinal properties.

Materials and methods

The freshwater macroalgae, *Chara vulgaris*, *Cladophora glomerata* and *Spirogyra crassa* were collected from the river Swat and river Kabul flowing through the Charsadda district, KPK state of Pakistan (Fig. 1). Taxonomic identification of the macroalgae was carried out by Prof. Arshad Iqbal of the Botany Department, Islamia College University Peshawar with the help of Prescot., [21], Tiffany and Britton books [22]. A specimen of each macroalgae has been stored in the Herbarium of the Botany department, Islamia College University Peshawar, Pakistan.

Sample collection and extraction

The collected freshwater macroalgae were washed with tap water and then with distilled water to remove sand, mud and other weeds. The clean freshwater macroalgae plants were spread out on plain paper and shade dried at room temperature for about 10 days and then ground into fine powder using a mechanical grinder. This powder was dipped in methanol for 3–4 days with irregular mixing with a glass rod for extraction of secondary metabolites. The mixture was filtered to remove any solid particles. This filtrate was concentrated by removing the methanol through rotary evaporator. This procedure was repeated three times for full extraction from the macroalgae powder. A dark green colored thick slurry was obtained after complete elimination of methanol under reduced pressure using a rotary evaporator. The obtained extract was stored in a refrigerator until further use.

Gas chromatography–mass spectrometry (GC–MS) analyses

GC–MS analyses of the essential oils present in the n-hexane fraction of the three freshwater macroalgae was carried out on a GC–MS-QP2010 Plus, comprising an AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer instrument employing the following conditions. A column elite-1 fused silica capillary column (30 M length × 0.25 mm-inner diameter × 1 μM

Fig. 1 Map of the area from which the macroalgae were collected and used in this study

film thickness, composed of 100% dimethyl polysiloxane) was operated in electron impact mode at 70 eV. Helium gas (99.99%) was used as a carrier gas at a constant flow of 1 mL/min and an injection volume of 0.5 µL was employed (split ratio of 10:1), with an injector temperature of 250 °C and an ion-source temperature of 280 °C. The oven temperature was programmed from 110 °C (isothermal for 2 min), with an increase of 10 °C/min to 200 °C, then at 5 °C/min to 280 °C, ending with a 9 min isothermal period at 280 °C. Each mass spectrum was collected at 70 eV with a scan interval of 0.5 s and fragments from 50 to 450 Da. Total GC running time was 46.95 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The software used to handle the mass spectra and chromatograms was TurboMass Version 2.0.7.

Antibacterial activity

The methanolic extracts of the freshwater macroalgae were tested for antibacterial activity using the disk diffusion method [23]. Extract antibacterial action was tested against four different bacteria. These were Gram-positive *Staphylococcus aureus, Bacillus subtilis* and Gram-negative *Xanthomonas campestris, Ralastonia solanacearum*. Each tested bacterium was transferred aseptically with an inoculating loop to a test tube having 5 mL of sterilized deionized water. Enough inoculum was poured so that the turbidity equaled standards. The test tube suspension (1 mL) was added to 15 mL of nutrient agar before setting aside the seeded agar plate (9 cm in diameter) to solidify for 15 min. Three disks of Whatman's No. 1 filter paper, 6 mm in diameter, were used to test the antimicrobial activity. Three concentrations of 500; 1000 and 1500 ppm were made for the extracts and were added to each respective disk (the three disks were equidistance from one another). For a negative control, dimethyl sulfoxide (DMSO) was added to one of the control disks and augmentin was used as a positive control on the other disk. All the discs were left for evaporation in sterile conditions and when the discs were dry, they were placed on sterile agar petri dishes freshly inoculated with the tested bacterial cell suspension. The Petri dishes were incubated for 24 h at a temperature of 36 °C in an aerobic environment. After the incubation time period, confluent bacterial growth was monitored. The zone of inhibition was measured in mm [24].

Phytotoxicity assay

The methanolic extracts of the freshwater macroalgae were tested for their herbicidal action. *Lemna minor* plant was used as a testing subject [1]. A series of solutions were made from the methanolic extracts by dissolving in methanol (MeOH). They had concentrations of 100, 300 and 500 ppm. Then each was transferred to a glass plate and the solvent was allowed to evaporate overnight. The sterilized media (100 mL) was poured on the glass containing dried extract and 10 rosettes of *Lemna minor*, each containing three fronds, were placed on it. A negative control as well as a positive control (atrazine) was run in parallel under a constant supply of tungsten light at room temperature for 7 days. The growth of *Lemna minor* was determined by counting the number of effected fronds (appearance of yellow color). The phytotoxicity was recorded by contrast with the negative control [25].

Insecticidal assay

The methanolic extracts of the freshwater macroalgae were tested for their insecticidal activity against *Tribolium castaneum* (a common grain pest known as red flour beetle). Test samples were prepared by dissolving 20 mg of extract in 20 ml of $d.H_20$ to create a stock solution. The sample (1572.7 µg/cm^2) was loaded over filter paper of an appropriate size (9 cm) on Petri dish plates using a micropipette. The plates were left for 24 h to evaporate the solvent. A negative control was treated with solvent to determine the effect of the solvent. Another batch, supplemented with reference insecticide was also made. The next morning, 10 healthy and active insects of each species of the same size and age were added to each plate including control (methanol) and standard drug (dichlorovos as a standard insecticide of 393.17 µg/cm^2). Thereafter the plates were incubated in a growth chamber at 27 °C for 24 h with 50% relative humidity. All of them were kept without food for 24 h. Mortality counts were carried out after a 24 h exposure period [26]. For calculation, the number of surviving insects was counted and the mortality (%) was determined. Results were the mean of three different experiments.

Cytotoxicity assay

A cytotoxic bioassay was performed to screen for those bioactive compounds that inhibit cell functions [7]. The lethality of the extract was checked against brine shrimp larvae using the procedure described by Oley and coworkers [27, 28]. Brine shrimp napuli were hatched in brine (35% aqueous sea salt solution). 31 day hatched napuli were then added to vials containing different concentrations of the extract (100, 300 and 500 ppm) in 15 mL brine with 1% DMSO. The DMSO was used to increase the solubility of the extract in brine. Three replicates were used for each concentration; blank (15 mL brine with 1% DMSO) was also run as a negative control.

Fig. 2 GC–MS chromatogram of the methanolic extract of *Chara vulgaris*

The toxicity of each sample was compared with that of blank (brine solution without crude extract) [28].

Statistical analysis

All the experiments were performed in triplicate and standard deviations (SD) were calculated in MS Office Excel. The results are presented as mean value with ± SD.

Results and discussion

Gas chromatography–mass spectrometry (GC–MS)

Figure 2 shows the GC–MS chromatogram of the methanolic extract of *Chara vulgaris.* A number of chemical compounds can be observed at different retention times in the chromatogram. These includes 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-Pyran-4-one; (S)-(+)-2′,3′-Dideoxyribonolactone at around 7.5 min of retention time. 1-Nitro-2-octanone is present at a retention time of 11 min. A peak for Biofermin is observed at 14 min and then a large peak of phenylpropanolamine is present at 14.5 min. Alpha-limonene diepoxide; tetradecanoic acid; *p*-menthane-1,2,8,9-diepoxy; 4-acetoxyphenyl-3-morpholino-propan-1-one; n-hexadecanoic acid; dl-Phenylephrine peak is present at retention time of 24.5 min. Phenylephrine has many properties that includes as a decongestant in winter, and it is also used for mydriasis (pupil dilation). It is also used to enhance blood pressure, and to treat hemorrhoids. Hexahydropseudoionone (terpene ketone), which is a fragrant in perfumes and cosmetics, was also detected. The 1-docosene; octadecanoic acid and various types of esters were also present as represented by different peaks at the end of the chromatogram of methanolic extract of *Chrara vulgaris* (Fig. 2 and Table 1). These results show the presence of various terpenes, ketones, alkenes, amines, complex fatty acids and their methyl esters in the methanolic extract of *Chara vulgaris*.

Figure 3 and Table 2 show the GC–MS profile of the various phytochemicals present in the methanolic extract of *Cladophora glomerata*. There are several methyl esters of complex fatty acids, such as 9-hexadecanoic acid, 5-octadecanoic acid and their derivatives. Similarly, 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- and other related octadecatrienoic acids were observed in the chromatogram of the methanolic extract of *C. glomerata*. 3-Propanediol,2-hydroxymethyl-2-nitro; Uranone,5,6,7,7-tetrahydro-4,4,7 a-trimethy, (R)-; Z,Z,Z-1,4,6,9-Nonadecatetraene and other related alkenes are observed at various retention times in the GC–MS analysis of the methanolic extract of *C. glomerata*. Most of the compounds mentioned in Table 2 and detected in other fractions of *C. glomerata* have commercial value and that is why it is a good source of revenue if it is grown on a commercial scale [29]. The *C. glomerata* extract is rich in phenolics that have high antioxidant activity [30], with the capability to reduce oxidative stress in mitochondria [31]. Similar compounds have been reported from *Cladophora* extract through GC–MS and were observed to have antifungal properties [32]. It has also been observed that when these extract are combined with nanoparticles their antifungal activity enhances [33].

The GC–MS chromatogram of *Spirogyra crassa* showed methyl esters of different complex fatty acids,

Table 1 Quantitative GC–MS analysis of methanolic extract of *Chara vulgaris*

ID#	Name of compound	R. time (min)	Area	Conc. (%)
1	Propylene carbonate	13.412	70,990	1.21
2	Benzene methanol, alpha-(1-aminoethyl)-[R-(R*,R*)]-	14.620	3,72,196	6.32
3	Methyl tetradecanoate	25.591	87,808	1.49
4	5-Isopropyl-6-methyl-hepta-3,5-dien-2-ol	21.485	92,257	1.57
5	1-Octadecyne	23.039	67,429	1.15
6	Hexahydropseudoionone	23.142	1,48,738	2.53
7	Pentadecanoic acid	23.393	10,539	0.18
8	(2E)-3,7,11,15-Tetramethyl-2hexadecen-1-ol	23.912	26,673	0.45
9	Linolenic acid, methyl ester	24.103	22,935	0.39
10	Methyl(7E,10E,13E)-7,10,13-hexadecatrienoate	24.221	1,11,973	1.90
11	11-Octadecenoic acid, methyl ester	24.342	10,478	0.13
12	Methyl 9-cycloprophylnonanoate	24.532	41,508	0.71
13	Hexadecanoic acid, methyl ester	24.766	12,46,193	21.17
14	1,4,8-Cyclododecatriene	24.985	2,24,533	3.81
15	9-Hexadecanoic acid	25.050	2,79,819	4.75
16	*n*-Hexadecanoic acid	25.453	10,24,948	17.41
17	(9E,12E,15E,)-9,12,15-Octadecatrien-1-ol	27.159	7988	0.14
18	Caprylic ether	27.337	19,441	0.33
19	Linolenic acid, methyl ester	27.692	19,979	0.34
20	Methyl(Z)-5,11,14,17-eicosatetraenoate	27.809	33,018	0.56
21	9,12-Octadecadienoic acid, methyl ester, (E,E)-	27.991	2,07,419	3.52
22	9,12-Octadecadienoyl chloride, (Z,Z)-	28.111	5,68,327	9.65
23	10-Octadecynoic acid, methyl ester	28.215	47,501	0.81
24	Phytol	28.355	4,62,143	7.85
25	Octadecynoic acid, methyl ester	28.605	1,44,147	2.45
26	9,12-Octadecadienoic acid, methyl ester, (E,E)-	28.674	1,29,180	2.19
27	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	28.808	3,12,003	5.30
28	Octadecanoic acid	29.261	29,697	0.50

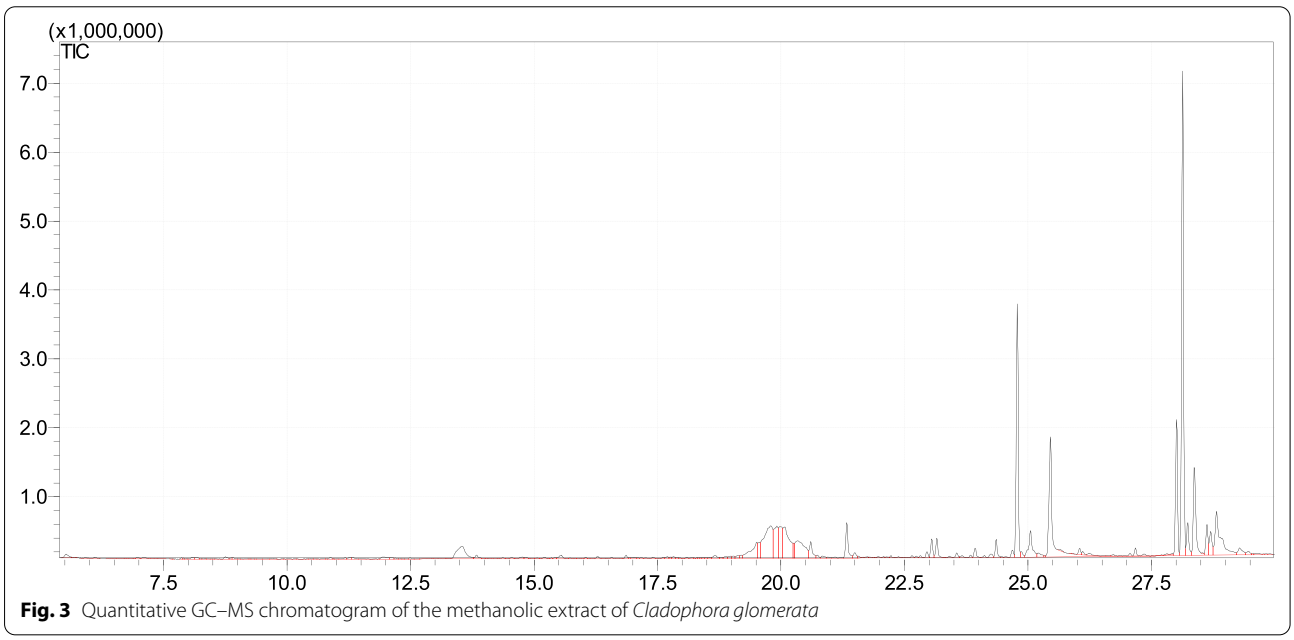

Fig. 3 Quantitative GC–MS chromatogram of the methanolic extract of *Cladophora glomerata*

Table 2 Quantitative GC–MS analysis of methanolic extract of *Cladophora glomerata*

ID#	Name of compound	R. time	Area	Conc. (%)
1	1,3-Propanediol,2-(hydroxymethyl)-2-nitro-	13.548	4,37,710	5.62
2	2(4H)-Benzofuranose,5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	16.285	12,367	0.16
3	Dodecanoic acid	16.863	15,169	0.19
4	Methyl tetradecanonate	20.603	1,01,327	1.30
5	Tetradecanoic acid	21.335	1,76,076	2.26
6	1-Octadecyne	23.052	68,907	0.89
7	2-Undecanone,6,10-dimethyl-	23.156	1,53,684	1.97
8	1-Octadecyne	23.557	20,549	0.26
9	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	23.927	29,928	0.38
10	9-Hexadecenoic acid, methyl ester, (Z)-	24.358	72,482	0.93
11	5-Octadecenoic acid, methyl ester	24.687	24,843	0.32
12	Hexadecenoic acid, methyl ester	24.532	41,508	0.71
13	Hexadecanoic acid, methyl ester	24.783	21,11,096	27.12
14	*n*-Hexadecanoic acid	25.456	7,42,555	9.54
15	Z,Z,Z-1,4,6,9-Nonadecatetraene	27.071	9939	0.13
16	1,4,8-Dodecatriene, (E,E,E)-	27.178	36,437	0.47
17	9,12-Octadecadienoic acid, methyl ester (E,E)-	28.010	5,17,411	6.65
18	9-Octadecanoic acid	28.134	16,47,973	21.17
19	10-Octadecanoic acid, methyl ester	28.234	1,42,577	1.83
20	Phytol	28.370	5,73,746	7.37
21	Octadecanoic acid, methyl ester	28.623	2,60,251	3.34
22	9,12-Octadecadienoic acid, methyl ester (E,E)-	28.698	1,43,532	1.84
23	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	28.819	3,11,027	4.00

such as decanoic acid; hexa and octa-decenoic acids and their various types (Fig. 4; Table 3). Like other GC–MS of macroalgae, phytol was also present in it. 1-Octadecyne is also present in *Spirogyra* extract.

Biological activities of the methanolic extracts

The methanolic extracts of the freshwater macroalgae were tested for biological activity by four different methods.

Antibacterial activity

The methanolic extracts of three freshwater green macroalgal species—*Chara vulgaris, Cladophora glomerata* and *Spirogyra crassa*—were tested against two Gram-positive and two Gram-negative species of bacteria. The methanolic extracts of all three species showed strong to moderate antibacterial activity against all four tested bacterial organisms (Table 4). The *C. vulgaris* methanolic extract showed weak activity against *B. subtilis*. The *C. glomerata* and *S. crassa* extracts showed moderate antibacterial action against *B. subtilis* as comapred to the standard drug augmentin. All three macroalgae extracts showed weak antibacterial activity against *Styphylococus aureus* at three different concentrations as compared to the standard antibiotic augmentin. When extracts were tested against the Gram-negative bacteria, *Xanthomonas compestris,* they showed weak activity as compared to the standard drug augmentin. All three macroalgae methanolic extracts showed moderate antibacterial action against the Gram-negative bacteria, *Ralastonia solanacerum*. Thus, these extracts showed weak antimicrobial activities. Several other investigators showed similar bactericidal activities from the extract of these and related macroalgal species. The methanolic extract of *Chara vulgaris* and its ethanolic-soluble part were active against a variety of Gram-positive and Gram-negative bacteria [34]. The aqueous extract of *Chara globularisis* was reported to show antibacterial activity against a natural population of bacteria from pond water [35]. The sterols extracted from *Chara wallichii* exhibited bioactivity against several species of bacteria [36]. This indicates that freshwater green algae may also display antibacterial activity, and consistently all of them are weakly active in our work (Table 4). The methanolic extract of *C. glomerata* has moderate antibacterial activity against the multidrug resistant *Acinetobacter baumannii* and several other pathogens [37]. The extract from *C. glomerata* also have antibacterial action against both Gram-positive and

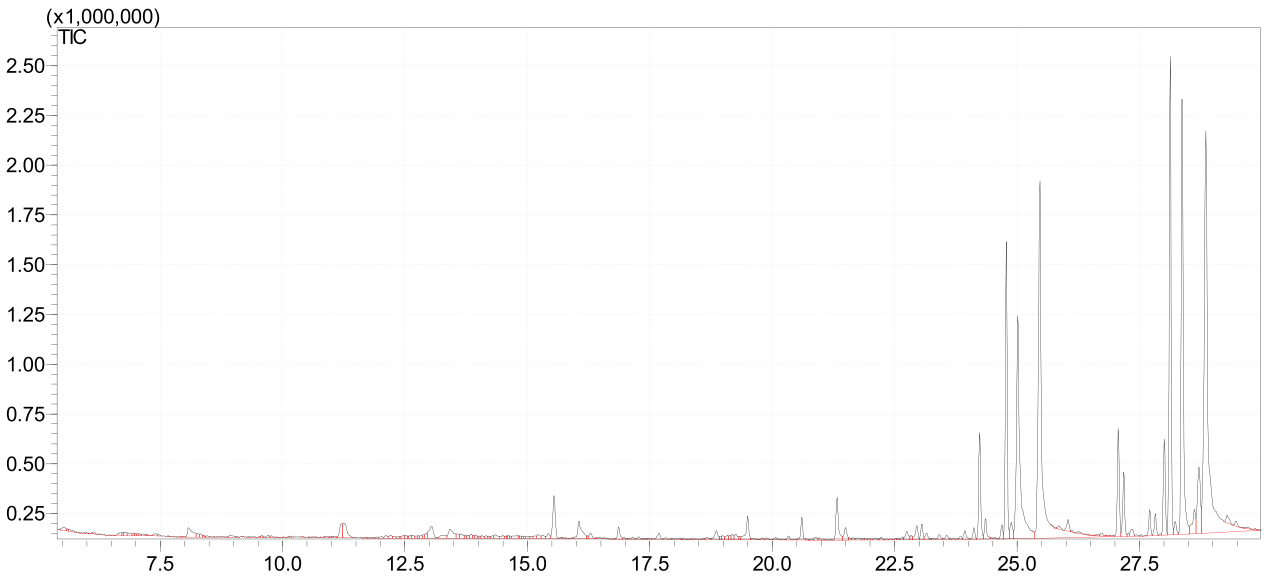

Fig. 4 GC–MS chromatogram of the methanolic extract of *S. crassa*

Table 3 Quantitative GC–MS analysis of methanolic extract of *Spirogyra crassa*

ID#	Name of compound	R. time	Area	Conc. (%)
1	Tridecanoic acid, methyl ester	16.045	37,156	0.81
2	Undecanoic acid	16.861	18,084	0.40
3	Methyl tetradecanoate	20.603	65,548	1.43
4	7-Oxabicyclo[4.1.0]heptane,1-methyl-4-(2-methyloxiranyl)-	21.496	29,890	0.65
5	Decanoic acid, methyl ester	22.737	13,925	0.30
6	1-Octadecyne	23.053	21,791	0.48
7	2-Undecanone,6,10-dimethyl-	23.155	14,643	0.32
8	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	24.118	10,503	0.23
9	7,10-Hexadecadienoic acid, methyl ester	24.117	15,866	0.35
10	9-Hexadecenoic acid, methyl ester, (Z)-	24.357	26,471	0.58
11	6-Octadecenoic acid, methyl ester(Z)-	24.689	15,734	0.34
12	Hexadecenoic acid, methyl ester	24.780	8,73,551	19.12
13	*n*-Hexadecanoic acid	25.465	8,24,789	18.06
14	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	27.067	1,50,748	3.30
15	1-Pentanol,3-methyl-2propyl-	27.348	13,159	0.29
16	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	28.129	5,11,251	11.19
17	Phytol	28.371	9,57,524	20.96
18	9,12-Octadecadienoic acid, methyl ester (E,E)-	28.715	1,45,788	3.19
19	Cis,cis,cis-7,10,13-Hexadecatrienal	28.860	8,21,435	17.98

negative strains [38]. The *C. glomerata* extract also posses bactericidal potential against *Mycobacterium* species [39].

Insecticidal activity

All of the three algal extracts were tested for their insecticiadal activity against the insect pest *Tribolium castaneum*, and dichlorovos was used as a standard insecticide. *Chara vulgaris* showed weak insecticidal activity, while *Cladophora glomerata* and *Spirogyra*

Table 4 Antimicrobial activity of methanolic extract of *Chara vulgaris*, *Cladophora glomerata* and *Spirogyra crassa*

***Bacillus subtilis* (G +ve)**				***Staphylococcus aureus* (G +ve)**		
Sample	**Concentration (ppm)**			**Concentration (ppm)**		
	500	**1000**	**1500**	**500**	**1000**	**1500**
C. vulgaris	0	11.73 ± 0.25	13.17 ± 0.76	10.4 ± 0.2	12.1 ± 0.36	15.93 ± 0.21
C. glomerata	17.33 ± 0.15	19.03 ± 0.31	22.17 ± 0.21	0	10.33 ± 0.15	13.97 ± 0.57
S. crassa	15.17 ± 0.21	17.17 ± 0.35	23.17 ± 0.31	10.1 ± 0.1	11.77 ± 0.21	14.97 ± 0.15
Augmentin	32.07 ± 0.21	32.93 ± 0.57	33.17 ±	29.33 ± 0.70	32.83 ± 0.21	32.87 ± 0.23
***Xanthomonas compestris* (G −ve)**				***Ralastonia solanacerum* (G −ve)**		
	Concentration (ppm)			**Concentration (ppm)**		
	500	**1000**	**1500**	**500**	**1000**	**1500**
C. vulgaris	–	10.83 ± 0.76	13.17 ± 0.76	–	11.17 ± 0.29	11.5 ± 0.5
C. glomerata	17.2 ± 0.2	20.1 ± 0.2	24.87 ± 0.25	–	11.73 ± 0.38	13.57 ± 0.51
S. crassa	13.23 ± 0.15	17.3 ± 0.4	21 ± 0.4	10.1 ± 0.1	12.37 ± 0.42	11.17 ± 0.58
Augmentin/+ C	33.1 ± 0.1	33.03 ± 0.30	33.1 ± 0.1	30.07 ± 0.21	30.83 ± 0.91	33.17 ± 0.21

The values show the zone of inhibition in millimeters, as detailed in the materials and methods. Augmentin was used as a standard drug, while DMSO was used as a negative control

Table 5 Insecticidal activity of crude methanolic extract of selected freshwater green macroalgae

Sample	**500 ppm**	**1000 ppm**	**1500 ppm**
C. vulgaris	2.33 ± 0.60	5.33 ± 1.53	5.33 ± 1.53
C. glomerata	5.33 ± 0.60	6.33 ± 0.58	6.33 ± 0.58
S. crassa	5.00 ± 1.00	6.66 ± 1.53	6.66 ± 1.53
Dichlorovos	7	10	10

Table 6 Phytotoxic activity of freshwater macroalgae methanolic extract on Lemna minor

Sample	**Concentration (ppm)**		
	100	**300**	**500**
C. vulgaris	2.33 ± 0.58	3.33 ± 0.58	2.67 ± 1.15
C. glomerata	3.67 ± 0.58	3.67 ± 0.58	5.67 ± 1.15
S. crassa	6.33 ± 1.53	6.33 ± 1.53	6.33 ± 0.58
Atrazine (herbicides)	5 ± 1	7.33 ± 0.58	8.33 ± 0.58
Control (water)	1.67 ± 0.58	0.33 ± 0.58	0.33 ± 0.58

crassa showed moderate to high insecticidal potential at 500, 1000 and 1500 ppm concentration (Table 5). Thus, *C. glomerata* and *S. crassa* contained compounds that are toxic to pests that destroy wheat and other grains. In the past, several algal extract studies showed insecticidal properties, for example *Chara globularis* has been reported to contain compounds with insecticidal properties [40]. The *C. glomerata* extract also possesses antiparasitic properties and is active against human vaginal parasites [41]. The phenolic extract of *Chara vulgaris* has more insecticidal potential than the standard drug albendazole against larva (Cysticercus) of *Taenia taeniaeformis* in rats [42]. Ethanolic extract of *C. vulgaris* has low insecticidal activity against the cotton leafworm *Spodoptera littoralis*, while *C. glomerata* has moderate activity [43].

Phytotoxic activity

The methanol extracts obtained from three species of freshwater green macroalgae were tested against *Lemna* spp. for phytotoxic activity. The *Spirogyra crassa* showed almost equal phytotoxic activity, such as the standard drug atrazine at all three concentrations tested (Table 6). *C. glomerata* extract also showed moderate phytotoxic activity at 100, 300 and 500 ppm. While the *Chara vulgaris* extract has poor phytotoxic activity. Most of the investigated algal species showed more than 50% phytotoxic activity against *L. minor*, with only *Cladophora glomerata* showing lesser activity (50%). Previous work has also shown that the methanol and ethanol extracts along with isolated sterols of *Chara wallichii* also exhibited significant phytotoxic activity against *L. minor* [36]. These results suggest that crude methanolic extracts need to be further evaluated for purified compounds that have powerful phytotoxic action.

Cytotoxic bioassay

Possible cytotoxic effects of the methanolic extracts of the three freshwater macroalgae were investigated against brine shrimp larvae. All three investigated extracts

Table 7 Cytotoxic activity of methanolic extract of selected freshwater macroalgae on brine shrimp

Sample	Concentration (ppm)		
	100	300	500
C. vulgaris	13.33 ± 1.25	16.33 ± 1.25	17.5 ± 0.5
C. glomerata	14 ± 2.16	15.33 ± 1.69	18 ± 0.82
S. crassa	13.67 ± 1.25	16 ± 1.63	16.67 ± 0.47
DMSO	0.0	0.0	0.0

displayed non-significant cytotoxic activity against brine shrimp (Table 7). Macroalgae contain several compounds that can be toxic to other cells and can inhibit the function of cells. Cytokinins—known to be ubiquitous among higher plants—have been isolated from *Chara globularis* [44]. Abscisic acid (ABA) has been detected in *C. foetida* [45], and sterols present in *C. coralline* were found to exhibit cytotoxic activity [34]. These observations indicate that freshwater green macroalgae may possess some cytotoxic substances. The methanolic extract was also tested for cytotoixc activity against human breast cancer cell lines and it was observed that it has no inhibitory action against cancer in this context [46]. However, when silver nanoparticles containing Cladophora extract are made they have suitable cytotoxic properties against colon cancers [47]. The *C. golmerata* was shown to be non-cytotoxic in in vivo studies [48].

Conclusions

GC–MS investigation of methanolic extract of the freshwater macroalgae *Chara vulgaris*, *Cladophora glomerata* and *Spirogyra crassa* from the Swat and Kabul rivers revealed that there are a number of different valuable compounds present in them. These compounds have medicinal, nutraceutical, cosmeceutical, and agricultural importance. Although our results showed weak bactericidal and cytotoxic activities, they have phytotoxic and insecticidal properties, suggesting that several compounds present in these methanolic extracts can be used in the agricultural sector. Therefore, these macroalgae may be part of the development of traditional medicines, nutraceuticals and food in the agricultural industry. The different compounds can be used as pesticides and insecticides. The biomass can be utilized for biofuel purposes. Further investigation is needed to isolate novel active compounds from these and other freshwater macroalgae from the Charsadda district, which may help in the development of algal farms on river banks.

Acknowledgements
We are thankful to the Higher Education Commission of Pakistan for their financial support to conduct this research study.

Authors' contributions
ZS performed the experiments with SB under the supervision of AI. The initial manuscript was written by ZS, AI and SB and MJ refined it and provided valuable comments. A-HE helped in interpretation of GC–MS analyses. All authors read and approved the final manuscript.

Funding
Higher Education Commission of Pakistan SRGP Grant.

Availability of data and materials
Available upon request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
All the authors declare that they have no competing interests.

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Received: 17 July 2021 Accepted: 14 November 2021
Published online: 15 January 2022

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