GC-MS based metabolomics and lipidiomics analyses of selected freshwater green macroalgae

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Abstract

Background

Macroalgae are a versatile source of biological molecules. The freshwater macroalgae can be used in a number of ways. They are a useful flora of fresh water bodies. They are cost effective remedy for the mitigation of excess phosphorus, nitrogen and other harmful chemicals in industrial wastewater. Here, the methanolic extracts of three freshwater macroalgae were further fractioned based on polarity of organic solvents and they were analyzed through gas chromatography-mass spectrometry.

Results

The gas chromatography-mass spectrometry based metabolomics and lipidomics identification of various fractions showed the presence of different important compounds. These includes long chain fatty acids, tepenes, ketones, alkaloids, hydrocarbons and benzene containing compounds. Several of these compounds have important medicinal and nutraceutical properties.

Conclusions

A number of useful fatty acids and other hydrocarbons were reported from these macroalgae. These lipids and hydrocarbons can be used in the production of biodiesel. This study provide a complete profiling of the various secondary metabolites present in the freshwater macroalgae *Chara vulgaris*; *Cladophora glomerata* and *Spirogyra crassa*.

Introduction

The word “macrophytes” is used for all filamentous algae since they can be seen forming masses floating on water surface and hence easily be harvested from its habitat. From ecological point of view, algae are widespread and present both in marine and fresh water habitats performing photosynthesis and assimilation of carbon dioxide [1]. The phycochemical agents synthesized by algae as a result of their metabolic activities generally include neutral lipids (fatty acids and Esters), polar lipids, fatty alcohols, prenylated moieties like terpenes, carotenoids, pyrrole derivatives like chlorophylls, phenolic compounds, ketones, alkanes and alkaloids [2–5]. From earlier biochemical investigation conducted on marine macroalgae showed that the natural products present have different important biological activities with promising results include antimicrobial; anticancer; antiviral; antimutageneic and antioxidative [6, 7]. The versatile nature of the biochemicals present in algae showed a number of medicinal properties that includes antimicrobial, antioxidant, anti-inflammatory, antiviral and antitumor bioactivities [8]. The freshwater algae are considered as a promising source of bioactive compounds [9]. The freshwater macroalgae are relatively unexplored as compared to marine algae considering chemical composition of compounds, which they produced. Although a number of taxonomical studies have been

conducted on green algae of Pakistan and its neighboring countries but insufficient biochemical data is available [10]. The marine algae present on coastal areas of Karachi has been investigated for various phycochemicals and their medicinal properties [11, 12]. The brackish water green algae is also investigated for its useful phycochemicals. It was observed that *Chara wallichii* A. Br. contain important sterol based compounds with antimicrobial and phytotoxic properties [13].

Freshwater green macroalgae *Chara vulgaris*, *Cladophora glomerata* (Linnaeus) KÜtzing and *Spirogyra crassa* are abundantly present throughout the year in river Swat, river Kabul and their tributaries flowing through district Charsadda, Pakistan. *Cladophora* is a ubiquitous group of reticulated filamentous green macroalgae. *C. glomerata* is a freshwater algae and it is used in many dishes as a dry sheets in Japan. *Cladophora* contains 200 recorded species that are present in freshwater and also in marine environment across the globe [14]. Many studies were conducted to explore its diversity and ecological importance but less attention have been given to highlight their phycochemical composition through GC-MS technique. In this work the phycochemical constituents were investigated in these three filamentous freshwater green macroalgae *Chara vulgaris*, *Cladophora glomerata* and *Spirogyra crassa* using gas chromatography coupled with mass spectrometry (GC-MS) analyzer to detect the different bioactive compounds. From which it will be easy to determine their biological, pharmacological, neutraceutical, dietary supplement and industrial importance. It is expected that this study will provide useful information for future extraction of phytochemicals from these macroalgae species of this area.

Methodology

2.1 Macroalgae Collection

The three filamentous, freshwater green macroalgae i.e *Chara vulgaris*, *Cladophora glomerata* and *Spirogyra crassa (Chlorophyta)* were collected from the river swat, river Kabul and their tributaries in district Charsadda, Khyber Pakhtunkhwa, Pakistan in the month of May to July 2020. The collected macroalgae were washed with normal water thrice and then with distill water to get rid of epiphytes and non-living matter. Taxonomic identification of the these macro-algae were carried out with the help of scientific literature and a Voucher specimen of each macroalgae were deposited and stored in the Herbarium of the Department of Botany, Islamia College University, Peshawar, Pakistan.

2.2 Sample preparation and extraction

After identification, all the three clean macroalgae biomass were shade dried separately for seven days on plain paper. The dried macroalgae were mechanically grinded by home electric grinder into powder form to facilitate extraction process and were stored in air tight glass jars for future extraction processes.

The lipids and secondary metabolites extraction from algal powder was carried out through putting the macroalgae powder in methanol for 3-5 days at 25 °C. The process of extraction was repeated two or more time to ensure complete extraction process. The extract of each macroalgae was then filtered with muslin cloth first and then through Whatman filter paper so that extract is free from solid particles. The filtered extract was concentrated through rotary evaporator by removing the methanol. A dark green colored slurry type crude extract was isolated after elimination of methanol under low pressure at 60 C° using rotary evaporator. The obtained extract from each respective macroalgae were weighted, labelled and stored at 4 °C.

2.3 Fractionation of crude Methanolic extracts

The obtained methanolic extract of the three freshwater green macroalgae were further fractionated separately one by one by first dissolving in water and when the two separate layers were formed. The upper hydrophobic layer was collected and further fractioned using organic solvents of increasing polarity from n-hexane, ethyl acetate to n-butanol. To start fractionation, the methanolic extract was resuspended in a mixture of distilled water and methanol, which were in a ratio of 70 mL: 30 mL. This sample was added to a separatory funnel and shake very well so, that methanolic extract dissolved in distilled water. 30 ml of n-hexane was supplemented to it, shake in only one direction, clock or anticlock wise direction three times and kept for ten minutes. The water layer was below because of high density and n-hexane layer was on the top of it. The n-hexane layer was collected from the water fraction and was named as n-hexane fraction. Then the same steps were followed for other solvents i.e. ethyl acetate and n-butanol by solvent-solvent extraction we start with the least order of polarity e.g we suspend the extract in water and portioning with n-hexane > ethyl acetate > n-butanol.

Each solvent fraction was isolated and placed in small beakers, and the solvent present in each fraction was evaporated by placing the respective fraction beaker in water bath at 60 C°. They were further dried by putting the sample in a desiccator. Fractions isolated from all three freshwater green macroalgae were subjected for further analysis to detect the essential metabolites through GC-MS technique.

2.4 Gas chromatography-mass spectrometry

The three different fractions of each macroalgae were tested through GC-MS for the presence of various compounds. For GC-MS, the same procedure was used as previously described. The chromatograms obtained were compared with the available library of chemical agent present in the GC-MS library of compounds.

Results

The GC-MS chromatogram of n-hexane fraction of *Chara vulgaris* are presented in **Figure 1.** The different phycochemicals identified from this fraction are tabulated in **Table 1**. GC-MS chromatogram revealed 15 main compounds existed in n-hexane fraction in which 1,3-benzene dicarboxylic acid bis (2-ethyl hexyl) ester; Bis-(2-ethylhexyl) phthalate and 1-docosene were abundantly present. The rest of the phycochemicals included long chain fatty acids, alkanes and benzene-containing molecules exists in the n-hexane extract of *C. vulgaris*.

Table 1. Quantitative result of the compounds identified in n-hexane fraction of *C. vulgaris*.

ID#	Name of Compound	Retention Time	Area	Conc. (%)
1	1,3-Benzene dicarboxylic acid, bis(2-ethylhexyl) ester	32.23	115396	4.42
2	Bis(2-ethylhexyl) phthalate	27.69	2029201	10.10
3	1-Docosene;	18.91	114961	0.21
4	Octadecanoic acid, methyl ester (Stearic acid)	17.31	107464	0.21
5	9-Octadecenoic acid, methyl ester, (E)-(Elaidic acid)	16.60	20670632	0.72
6	1-Eicosanol	13.98	76018	0.42
7	Hexadecanoic acid, methyl ester (Palmitic acid)	12.50	68613	1.27
8	2-Pentadecanone, 6,10,14-trimethyl-	10.77	311342	0.24
9	Tetradecanoic acid, methyl ester (Myristic acid)	8.59	94727	0.21
10	Phenol, 2,4-bis(1,1-dimethylethyl)-	5.99	891654	0.02
11	Tridecane	4.59	44577	0.06
12	Undecane	3.42	142649	0.09
13	Decane	2.78	105425	0.24
14	p-Xylene	2.80	294836	0.61
15	o-Xylene	1.92	34455	0.06

The chromatogram of n-hexane portion of *Cladophora glomerata* is shown in **Figure 2**. The different phycochemicals identified from this fraction are tabulated in **Table 2**. The results revealed fifteen major compounds in n-hexane portion, in which and 1,3-Benzene dicarboxylic acid bis (2-ethylhexyl) ester; Bis (2-ethylhexyl) phthalate and 1-tetracosanol were abundantly present. The long chain fatty acids includes hexadecanoic acid, 9-Octadecenoic acid methyl ester and several long chain alkane molecules were present in *C. glomerata* n-hexane extract.

Table 2. Quantitative result of the compounds identified in n-hexane portion of *C. glomerata.*

ID#	Name of Compound	Retention Time	Area	Conc. (%)
1	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	32.31	115396	4.42
2	Bis(2-ethylhexyl) phthalate (Phthalic acid)	27.79	2029201	10.10
3	1-Tetracosanol (Tetracosane)	23.95	114961	0.10
4	Oleic Acid, methyl ester (Oleic acid)	19.08	107464	0.01
5	9-Octadecenoic acid, methyl ester, (E)-(Elaidic acid)	16.61	20670632	0.31
6	1-Eicosanol (Arachidyl alcohol)	14.00	76018	0.42
7	n-Hexadecanoic acid (Palmitic acid)	13.67	68613	1.09
8	Hexadecanoic acid, methyl ester (Palmitic acid)	12.52	311342	1.27
9	2-Pentadecanone, 6,10,14-trimethyl-(Fitone)	10.77	94727	0.24
10	Methyl tetradecanoate (Methyl myristate)	8.59	891654	0.21
11	2,5-Cyclohexadiene-1,4-dione, (Benzoquinones) 2,6-bis(1,1-dimethylethyl)-	5.64	44577	0.04
12	Tridecane	4.59	142649	0.06
13	Undecane	3.42	105425	0.09
14	Undecane	2.78	294836	0.24
15	o-Xylene	1.92	34455	0.61

The GC-MS chromatogram of n-hexane portion showed fatty acid methyl esters (FAME) of *Spirogyra crassa* (**Figure 3)**. While the different Fatty acids identified from this fraction are tabulated in **table 3**. GC-MS results revealed 21 FAMEs identified from n-hexane fraction in which Palmitate methyl ester and Linolate methyl ester were abundantly present. Myristic acid methyl ester, Lauric acid methyl ester, Stearic acid methyl ester were moderately abundant. 4,7,10,1316,19-Docosahexaenoic acid methyl ester, 11-hexadecanoic acid methyl ester were present in lower amount. Other minor compounds were arachidonic acid methyl ester, Oleic acid methyl ester, pentadecanoic acid methyl ester, 5,8,11,14,17-Eicosapentaenoic acid methyl ester, elaidic acid methyl ester, capric acid methyl ester, margaric acid methyl ester, caprylic acid methyl ester, arachidic acid methyl ester, Linoleic acid methyl ester, Linoleic acid methyl ester, hexonic acid methyl ester and undecanoic acid methyl ester.

Table 3. Compounds identified in the n-hexane portion of *S. crassa.*

ID#	Name of Compound	R.Time	Area	Conc. (%)
1	Hexonic acid methyl ester	4829	11435	0.04
2	Caprylic acid methyl ester	10.005	73831	0.24
3	Capric acid ,methyl ester	15.474	93476	0.30
4	Undecanoic acid methyl ester	18.053	2613	0.01
5	Lauric acid methyl ester	20.517	910939	2.90
6	Tridecanoic acid methyl ester	23.027	7763	0.02
7	Myristic acid methyl ester	26.003	1554298	4.95
8	Pentadecanoic acid methyl ester	29.402	214019	0.68
9	11-Hexadecanoic acid methyl ester	32.271	333998	1.06
10	Palmitic acid methyl ester	33.213	15601416	49.69
11	Margaric acid, methyl ester	37.645	93526	0.30
12	Arachidonic acid methyl ester	39.898	279256	0.89
13	Oleic acid, methyl ester	40.514	205183	0.70
14	Linoleic acid methyl ester	40.740	7854678	26.95
15	Elaidic acid methyl ester	40.897	99523	0.34
16	Stearic acid methyl ester	41.483	868984	2.77
17	5,8,11,14,17-Eicosapentaenoic acid methyl ester	43.907	211779	0.67
18	4,7,10,1316,19-Docosahexaenoic acid methyl ester	44.024	585617	1.87
19	Linoleic acid methyl ester	44.293	35627	0.11
20	Linoleic acid methyl ester	44.705	53158	0.17
21	Arachidic acid methyl ester	45.442	60971	0.19

The ethyl acetate portion of *Chara vulgaris* is shown in **figure 4,** while the compounds identified from this fraction are presented in **table 4**. GC-MS results revealed 21 phycochemical compounds identified in ethyl acetate fraction in which n-hexadecanoic acid (Palmitric acid), 9-Octadecenoic acid methyl ester (E) (Elaidic acid), hexadecenoic acid methyl ester were major part of the chromatogram. Benzeneethalnamine N, alpha-dimethyl-(S); 5-Isopropyl1-6-methyl-hepta-3,5-dien-2-ol; 2-(4-Hydrophenyl) ethylformamide; 2-Undecanone,6,10-dimethyl; 9, 12 Octadecadienoic acid methyl ester, (E,E) (Linoleic acid); 7-Tetradecenal (Z) were moderately abundant. Tetradecanoic acid (Myristic acid); Octadecanoic acid methyl ester (Stearic acid); dl-Phenylephrine; 10-Octadecenoic acid methyl ester; 3-Buten-2-one,4-(4-hydroxy-2,2,6-trimethyl 1,7-oxabicyclo were present in lower amount. Other minor compounds were 7-Oxabicyclo[4.1.0]heptane,1-methyl-4-2(2-methyloxiranyl); Tetradecanoic acid (Myristic acid); 9-Hexadecanoic acid; Dodecanoic acid (Lauric acid); 1,1-(4-methyl1-1,3-phenylene)bis{3-[5-(p-tolyl]-1,3,4-thiadiazol-2 and Tetradecanoic acid.

Table 4. GC-MS chromatogram analysis of ethyl acetate fraction of *C. vulgaris*.

ID#	Name of Compound	R. Time	Area	Conc. (%)
1	Benzeneethanamine, N, alpha-dimethyl-(S)	8.01	856861	9.72
2	Undecanoic acid	16.87	32376	0.37
3	3-Buten-2one,4-(4-hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0]	19.79	100875	1.14
4	7-Oxabicyclo[4.1.0]heptane,1-methyl-4-(2-methyloxiranyl)	20.88	87452	0.99
5	Tetradecanoic acid	21.34	221210	2.51
6	5-Isopropyl-6-methyl-hepta-3,5-dien-2-ol	21.51	512559	5.81
7	2-(4-Hydroxyphenyl) ethyl formamide	22.89	484384	5.49
8	2-Indecanone,6,10-dimethyl	23.16	301686	3.42
9	Tetradecanoic acid	23.41	14594	0.17
10	dl-Phenylephrine	23.96	146442	1.66
11	9-Hexadecanoic acid, methyl ester,(Z)	24.36	36098	0.41
12	Hexadecenoic acid	24.78	1499329	17.01
13	9-Hexadecanoic acid	25.05	106405	1.21
14	n-Hexadecanoic acid(SFA,Palmitric acid)	25.49	2128519	24.14
15	1,1’-(4-Methyl-1,3-phenylene)bis{3-[5-(p-tolyl)-1,3,4-thiadiazol-2	27.81	31176	0.35
16	9,12-Octadecadienoic acid methyl ester (E,E)	28.01	307656	3.49
17	9-Octadecadienoic acid,methyl ester (E)	28.13	1313467	14.90
18	10-Octadecenoic acid, methyl ester	28.23	106718	1.21
19	Octadecanoic acid, methyl ester	28.62	206593	2.34
20	7-Tetradecenal,(Z)	28.81	265446	3.01
21	Tetradecanoic acid	29.28	56488	0.64

The chromatograms of ethyl acetate portion of *Cladophora glomerata* are shown in **Figure 5** and the compounds recognized from this fraction are tabulated in **Table 5**. GC-MS data showed 27 phycochemical compounds recognized from ethyl acetate portion in which Pentadecanoic acid; 14-methyl-,methyl ester/Pentadecyclic acid; Phytol; 6-Octadecenoic acid,methyl ester; (Z)-(12.07%),5-Isopropyl-6methyl-hepta-3,5-dien-2-ol were abundantly present. (3E)-4-(4-Hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1yl)-3; 9,12-Octadecadienoic acid methyl ester; (E,E)-Oleic acid; 2-Undecanone,6,10-dimethyl; (+)-(S)-Deoxyephedrine; Octadecanoic acid were moderately abundant. Tridecanoic acid methyl ester; 11-Octadecenoic acid methyl ester; 1-Nitro-2-octanone; 1-Nitro-2-octanone; dihydro actinidiolide; 5-Cyclooctene-1,2-dione; Cyclohexyl isoprophyl phosphonolfluoridate; 1-Octadecyne; Tetradecanoic acid were present in lower amount. Other minor compounds were 1-Hepten-6-one,2-methyl; 9-Hexadecenoic acid methyl ester, (Z); Thujol; Hexadecane; Phytol; 2-iodo-6-methylheptane; 7,10-Hexadecadienoic acid methyl ester; 1,5,9,11-Tridecatetraene,12-methyl-,(E,E).

Table 5. Compounds present in the chromatogram of ethyl acetate fraction of *C. glomerata*.

ID#	Name of Compound	R. Time	Area	Conc. (%)
1	(+)-(S)-Deoxyephedrine	8.03	341729	3.42
2	5-Cyclooctene-1,2-dione	8.91	118075	1.18
3	1-Nitro-2-octanone	10.88	133280	1.33
4	Cyclohexyl isoprophylphosphonolfluoridate	12.50	117923	1.18
5	1-Hepten-6-one,2-methyl	13.40	98980	0.99
6	Thujol	18.68	73925	0.74
7	(3E)-4-(4-Hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1yl)-3	19.79	565891	5.66
8	Hexadecane	20.07	54796	0.55
9	Tridecanoic acid methyl ester	20.60	146797	1.47
10	Dihydro actinidiolide	20.88	126763	1.27
11	Tetradecanoic acid	21.33	105168	1.05
12	5-Isopropyl-6methyl-hepta-3,5-dien-2-ol	21.52	813841	8.13
13	1-Octadecyne	23.05	107757	1.08
14	2-Undecanone,6,10-dimethyl	23.15	420215	4.20
15	Phytol	23.54	48576	0.49
16	7,10-Hexadecadienoic acid methyl ester	24.11	25347	0.25
17	9-Hexadecenoic acid methyl ester,(Z)	24.35	97853	0.98
18	Pentadecanoic acid,14-methyl, methyl ester	24.78	2284452	22.83
19	n-Hexadecanoic acid	25.46	918099	9.18
20	1,5,9,11-Tridecatetraene,12-methyl,(E,E)	27.17	27341	0.27
21	2-iodo-6-methylheptane	27.34	35300	0.35
22	9,12-Octadecadienoic acid methyl ester,(E,E)	28.0	452429	4.52
23	6-Octadecenoic acid methyl ester,(Z)	28.12	1207848	12.07
24	11-Octadecenoic acid methyl ester	28.22	138303	1.38
25	Phytol	28.36	1234976	12.34
26	Octadecanoic acid	28.61	310070	3.10

The ethyl acetate fraction chromatogram of *Spirogyra crassa* are presented in **figure 6** and the compounds recognized from this fraction are tabulated in **table 6**. There are 25 phycochemical compounds present in this fraction, in which benzene-ethanamine, N, alpha-dimethyl-(S); Phytol; n-Hexadecanoic acid; 9,12,15-Octadeccatrien-1-ol(Z,Z,Z); were abundantly present. 9,12,15-Octadecatrienoicc acid methyl ester(Z,Z,Z); Hexadecanoic acid methyl ester were moderately abundant. Octadecanoic acid ethyl ester, Sulfuric acid 5,8,11-heptadecatrienyl methyl ester; Tetradecanoic acid were present in lower amount. Other minor compounds were 5-Isppropyl-6methyl-hepta-3,5-dien-2-ol; dodecanoic acid; 2-Undecanone,6,10-dimethyl; 1-Octadecyne; 2(4H)-Benzofuranase,5,6,7a-tetrahydro-

4,4,7a-trimethyl-,(R); Methyl tetradecanoate; 7-Oxabicyclo[4.1.0]heptane,1-methyl1-4-(2methylloxiranyl); 1-Octadecyne; Cyclopropaneoctanoic acid 2-hexyl-methyl ester; Pentadecanoic acid; 3,7,11,15-Tetramethyl-2-hexadecen-1-ol; Decane,2,3,4,5,8-tetramethyl-; n-Butyl laurate; Docosanoic acid, ethyl ester; 7,10-Hexadecadienoic acid, methyl ester; 7-Hexadecenoic acid, methyl ester,(Z).

Table 6. Quantitative result table of GCMS analysis of ethyl acetate fraction of *S. crasssa.*

ID#	Name of Compound	R. Time	Area	Conc. (%)
1	Benzeneethanamine,N,alpha-dimethyl-(S)	8.21	5566020	30.57
2	2(4H)-Benzofuranase,5,6,7a-tetrahydro-4,4,7a-trimethyl-,(R)	16.28	45093	0.25
3	Dodecanoic acid	16.87	82424	0.45
4	Decane,2,3,4,5,8-tetramethyl	20.07	18724	0.10
5	Methyl tetradecanoate	20.59	35532	0.20
6	7-Oxabicyclo[4.1.0]heptane,1-methyl1-4-(2methylloxiranyl)-	20.88	28554	0.16
7	Tetradecanoic acid	21.32	191322	1.05
8	5-Isppropyl-6methyl-hepta-3,5-dien-2-ol	21.49	118358	0.65
9	n-Butyl laurate	21.89	16975	0.09
10	Docosanoic acid ,ethyl ester	22.07	17176	0.09
11	1-Octadecyne	23.04	53329	0.29
12	2-Undecanone,6,10-dimethyl	23.14	61158	0.34
13	Pentadecanoic acid	23.40	22459	0.12
14	1-Octadecyne	23.558	27265	0.15
15	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	23.92	19560	0.11
16	7,10-Hexadecadienoic acid ,methyl ester	24.11	17000	0.09
17	Cyclopropaneoctanoic acid ,2-hexyl-methyl ester	24.35	27291	0.15
18	7-Hexadecenoic acid, methyl ester,(Z)	24.68	9819	0.05
19	Hexadecanoic acid, methyl ester	24.77	603495	3.31
20	9,12,15-Octadeccatrien-1-ol(Z,Z,Z)	25.05	2463190	13.53
21	n-Hexadecanoic acid	25.51	3681462	20.22
22	Octadecanoic acid, ethyl ester	26.11	345564	1.90
23	Sulfuric acid,5,8,11-heptadecatrienyl methyl ester	27.06	320389	1.76
24	9,12,15-Octadecatrienoicc acid methyl ester (Z,Z,Z)	28.12	669170	3.68
25	Phytol	28.37	3763942	20.68

The chromatogram of n-butanol portion of *Chara vulgaris* are presented in **figure 7**, while the compounds recognized from this fraction are tabulated in **table 7**. GC-MS showed twenty-seven phycochemical compounds, which are extracted with n-butanol. In this fraction in phenylepropanolamine were abundantly present. (S)-(+)-2',3'-Dideoxyribonolactone; Hexadecanoic acid, methyl ester; 2- (4-Hydroxyphenyle1) ethyleformamide; 7-Hexadecenoic acid, methyl ester, (Z)- were moderately abundant. n-Hexadecanoic acid; p-Menthane, 1, 2:8, 9-diepoxy; D1-phenylephrine; 9,12-Octadecadienoic acid, methyl ester, (E,E)-; Octadecanoic acid, methyl ester; 2-Undecanone,6,10-dimeyhyle-; Phytol; 1-Heptadecanol were present in lower amount. Other minor compounds were, 3, 5-Dihydroxy-6-methyle-2,3—dihydro-4H-pyran-4-one; 1-Nitro-2-Octanone; Biofermin; Isobutyl-2-heptenone; 2-Methyle-Z,Z-3,13-octadecadienol; alpha-Limonene diepoxide; Octadecanoic acid; Tetradecanoic acid; 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione; 1-Docosene;7-Hexadecanoic acid, methyle ester, (Z)-.

Table 7. Compounds identified in the n-butanol fraction of *Chara vulgaris.*

ID#	Name of Compound	R. Time	Area	Conc. (%)
1	3,5-Dihydroxy-6-methyle-2,3—dihydro-4H-pyran-4-one	6.96	115396	0.40
2	(S)-(+)-2′,3′-Dideoxyribonolactone	7.84	2029201	7.05
3	1-Nitro-2-Octanone	10.88	114961	0.40
4	Biofermin (Tremorine)	13.83	107464	0.37
5	Phenylepropanolamine	14.49	20670632	71.81
6	alpha-Limonene diepoxide	20.89	76018	0.26
7	Tetradecanoic acid	21.33	68613	0.24
8	p-Menthane,1,2:8,9-diepoxy	21.50	311342	1.08
9	1-(4-Acetoxyphenyle)-3-morpholino-propan-1-one	22.26	94727	0.33
10	2-(4-Hydroxyphenyle1) ethyl formamide	22.95	891654	3.10
11	E-2-Tetradecen-1-01	23.04	44577	0.15
12	2-Undecanone,6,10-dimethyl	23.15	142649	0.50
13	Isobutyl-2-heptenone	23.59	105425	0.37
14	D1-phenylephrine	24.0	294836	1.02
15	7-Hexadecanoic acid, methyl ester,(Z)	24.35	34455	0.12
16	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	24.67	47613	0.17
17	Hexadecanoic acid, methyl ester	24.77	1077706	3.74
18	n-Hexadecanoic acid	25.45	821109	2.85
19	1-Docosene	26.11	48959	0.17
20	1-Heptadecanol	27.78	125061	0.43
21	9,12-Octadecadienoic acid, methyl ester,(E,E)	27.99	252449	0.88
22	7-Hexadecenoic acid, methyl ester,(Z)	28.12	864879	3.00
23	Phytol	28.36	125898	0.44
24	Octadecanoic acid, methyl ester	28.61	148430	0.52
25	2-Methyle-Z,Z-3,13-octadecadienol	28.79	102289	0.36
26	Octadecanoic acid	29.27	67149	0.23

The n-Butanol portion of *C. glomerata* are presented in **figure 8** and the compounds present in this fraction are tabulated in **Table 8**. There are 26 Phycochemical compounds identified from n-butanol fraction in which Hydroxymethylefurfurol (HMF); n-Hexadecanoic acid; Hexadecanoic acid, methyl ester; Phenylemalonic acid; 3,5-Dihydroxy-6-methyle-2,3-dihydro-4H-pyran-4-one were abundantly present. 9,12-Octadecanoic acid, methyl ester,(E); 2-Deoxyhexose,1-Nitro-2-octanone; 9,12-Octadecadienoic acid, methyl ester, (E,E)-; 3-Buten-2-one,4-(4-hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0] were moderately abundant. Octadecanoic acid, ethyl ester; 9-OHexadecanoic acid; Z-2-Octadecen-1-ol acetate; 1H-Purine-2, 6-dione, 3,

7-dihydro-1,3,7-trimethyl; 7-Oxabicyclo[4.1.0]heptane,1-methyl-4-(2-methylloxiranyl); 1-Heptadecanol; 1-Docosene were present in lower amount. Other minor compounds were 2-bromo Octane; 9,12,15-Octadecatrien-1-ol,(Z,Z,Z)-; 9,12-Octadecadienoic acid, methyl ester,(E,E)-; 13-Octadecanoic acid, methyl ester; 1-Docosene; 5,7-Octadien-2-one,3-acetyl-; Erucic acid; Phytol; 7,9-Di-ter-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dieone; 2-Cyclohexane-1-one,4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl; Cyclopropane octanoic acid,2-hexyl-, methyl ester; E-14-hexadecenal.

Table 8. Quantitative results of GCMS analysis of n-butanol fraction of *C. glomerata*.

ID#	Name of Compound	R. Time	Area	Conc. (%)
1	3,5-Dihydroxy-6-methyle-2,3-dihydro-4H-pyran-4-one	6.96	470726	5.28
2	Hydroxymethylefurfurol9	8.78	5136135	**57.66**
3	Phenyl malonic acid	9.25	512689	5.76
4	1-Nitro-2-octanone	10.88	173314	1.95
5	2-Deoxyhexose	14.08	205414	2.31
6	3-Buten-2-one,4-(4-hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0]	19.79	99360	1.12
7	7-Oxabicyclo[4.1.0]heptane,1-methyl-4-(2-methylloxiranyl)	20.89	54507	0.61
8	5,7-Octadien-2-one,3-acetyl	21.74	31365	0.35
9	2-Cyclohexane-1-one,4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl	21.98	16401	0.18
10	E-14-Hexadecenal	22.07	11442	0.13
11	1H-Purine-2,6-dione,3,7-dihydro-1,3,7-trimethyl	23.10	63862	0.72
12	Z-2-Octadecen-1-ol acetate	23.56	70169	0.79
13	Cyclopropaneoctanoic acid,2-hexyl-,methyl ester	24.35	14006	0.16
14	7,9-Di-ter-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dieone	24.66	17107	0.19
15	Hexadecanoic acid, methyl ester	24.77	529336	5.94
16	Erucic acid	25.02	23793	0.27
17	n-Hexadecanoic acid	25.43	575091	6.46
18	1-Docosene	26.11	37909	0.43
19	Octane,2-bromo	27.34	21716	0.24
20	1-Heptadecanol	27.78	47605	0.53
21	9,12-Octadecadienoic acid, methyl ester,(E,E)	27.99	147898	1.66
22	9,12-Octadecanoic acid, methyl ester,(E)	28.11	441507	4.96
23	13-Octadecanoic acid, methyl ester	28.21	41328	0.46
24	Phytol	28.36	23290	0.26
25	Octadecanoic acid, methyl ester	28.61	82563	0.93
26	9-OHexadecanoic acid	28.78	82415	0.93

The chromatogram of n-butanol portion of *S. crassa* are presented in **figure 9**. While the compounds recognized from this fraction are tabulated in **table 9**. GC-MS data showed 25 Phycochemical compounds recognized from n-butanol portion in which n-hexadecanoic acid; 9,12,15-Octadecadienoic acid, (Z,Z,Z)-; Phytol; Hexadecanoic acid, butyl ester were the main components. Hexadecanoic acid, methyle ester; 9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-; 9-Octadecynoic acid; 1,6-Anhydro-beta-D-glucopyranose

(levoglucosan) were moderately abundant. Octadecanoic acid, ethyl ester; 1,2,3-Benzenetriol; Sulfuric acid, 5,8,11-eptadecatrienyl methyl ester; 7-Hexadecanoic acid, methyl ester; Tetradecanoic acid; Tetradecanoic acid; 1-Di (tert-butyl) silyloxypentane were present in lower amount. Other minor compounds were 1,E-11,Z-13-Octadecatrien; 9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-; 9,12-Octadecadienoic acid, methyl ester,(E,E)-; Pentanoic acid, octyl ester; 7, 10, 13-Hexadecatrienoic acid, methyl ester; 4-Methylocatanoic acid; n-Decanoic acid; Pentadecanoic acid; Cyclopropaneoctanoic acid, 2-hexyl-methyl ester; dihydro actinidiolide and Nonanedioic acid, monomethyl ester.

Table 9. GCMS chromatogram of n-butanol fraction of *S. crassa*.

ID#	Name of Compound	R. Time	Area	Conc. (%)
1	1,2,3-Benzenetriol	12.38	145399	1.82
2	Actinidiolide, dihydro	16.283	7520	0.09
3	n-Decanoic acid	16.86	18197	0.23
4	Nonanedioic acid, monomethyl ester	17.39	5982	0.07
5	Tetradecanoic acid	21.32	138495	1.73
6	Pentanoic acid, octyl ester	22.52	33183	0.41
7	1,6-Anhydro-beta-D-glucopyranose	22.83	186417	2.33
8	1-Di(tert-butyl) silyloxypentane	23.10	80628	1.01
9	Pentadecanoic acid	23.39	18023	0.23
10	7,10,13-Hexadecatrienoic acid, mehyl ester	24.23	22610	0.28
11	Cyclopropaneoctanoic acid,2-hexyl-,methyl ester	24.35	10478	0.13
12	Hexadecanoic Acid, methyl ester	24.77	70169	3.84
13	1,E-11,Z-13-Octadecatrien	24.89	71476	0.89
14	9,12,15- Octadecatrien-1-ol,(Z,Z,Z)	25.03	1143538	14.30
15	n-Hexadecanoic acid	25.48	1731896	21.65
16	Sulfuric acid,5,8,11-heptadecatrienyl methyl ester	27.06	86814	1.09
17	9,12,15-Octadecatrien-1-ol,(Z,Z,Z)	27.17	59882	0.75
18	4-Methylocatanoic acid	27.34	19091	0.24
19	9,12-Octadecadienoic acid, methyl ester,(E,E)-	27.99	54514	0.68
20	7-Hexadecanoic acid, methyl ester	28.12	140079	1.75
21	Phytol	28.36	1085125	13.56
22	9-Octadecynoic acid	28.70	184509	2.31
23	9,12,15-Octadecadienoic acid,(Z,Z,Z)	28.86	1590-731	19.89
24	9,12,15-Octadecatrien-1-ol,(Z,Z,Z)	29.27	246971	3.09
25	Hexadecanoic acid, butyl ester	29.86	611036	7.64

Discussion

The GC-MS analysis in methanolic extracts of brown seaweed *Sargassum wightii* indicated the presence of 5-Isopropyl-6-methyl-hepta-3,5-dien-2-ol [15]. Thujone and thujol are isolated from *Salviae aetheroleum* extract, they are neurotoxic and abortive substances [16]. We also detected thujol in *C. glomerata*. Cyclohexyl-isopropyl-phosphono-flouridate (sulcatone) act as a insects repellent and can be used against

the increasingly adapting *Anopheles* and *Aedes* species of mosquitoes [17]. The terpenes were detected in all three freshwater macroalgae only in ethyl acetate and n-butanol fractions, but with different concentrations. The total terpene in these three green macroalgae was represented with peak are present mostly in *Cladophora* and *Spirogyra crassa* and represented by alpha-Limonene diepoxide; 3,7,11,15-Tetramethyl-2-hexadecen; actinidiolide and phytol. Phytol were the dominant terpene. Phytol is part of the chlorophyll molecule and present in plants and green algae [18, 19]. Phytol is also part of vitamin E and K [19]. The ionone derivatives, dihydro actinidiolide were noticed in marine and freshwater algae e.g. *Spirogyra* and *Cladophora* [20, 21]. The antibacterial potential of several furanone compounds e.g. dihydro actinidiolide, a volatile terpene are well established [22].

The fats are the reserves of energy for organisms beside allelophathic and antibacterial properties [23]. Living organisms convert acetyl co-A into fatty acids. The saturated fatty acids are linked to hypercholesterolemia and induce the expression of cyclooxygenase-2 [24]. It is known that fatty acids from freshwater algae possess antibacterial activity [9, 10, 25]. The n-butanol fraction has eight fatty acids represented by n-hexadecanoic acid and tetradecanoic acid in *Spirogyra crassa* followed by *Cladophora glomerata* with ethyl acetate having three saturated fatty acids and n-butanol fraction has three fatty acids and ethyl acetate fraction has six saturated fatty acids represented by n-hexadecnoic acid in n-butanol fraction having n-hexadecanoic acid in *Chara vulgaris*. The n-hexadecanoic acid and tetradecanoic acids were dominant saturated fatty acids present in all these three macroalgae in all fractions. Several fatty acids have antimicrobial potency, for example palmitic acid which is present in various fractions of *Chara vulgaris*. A similar study performed on *Bortryoccus braunii* fatty acids showed antimicrobial activity [26]. The extract of this algae is rich in fatty acids like linolenic, oleic, lanolin and n-hexadecanoic acid [26]. The fatty acid profile of *Spirogyra crassa* investigated here was in agreement with that reported in other species of *Spirogyra* [27]. Fatty alcohols are associated with plant and animal lipids and are utilized in pharmaceutics, detergents and plastics formation [5]. Fatty alcohols in these fresh water green macroalgae were represented by three saturated fatty alcohols like myristic alcohol, arachidyl alcohol, Linolenyle alcohol. The E-2-Tetradecen-1-ol; 1-Heptadecanol; 7-Tetradecenal, (Z)-myristic alcohol; 1-Eicosanol and arachidyl alcohol are also present. Fatty alcohols were present in the ethyl acetate and n-butanol fractions of these three green macroalgae with maximum in ethyl acetate fraction of *Chara vulgaris*. These long chain fatty alcohols were dominant alcohols in all three fractions, n-hexane, and ethyl acetate and n-butanol fractions in these green macroalgae. Two fatty alcohols, 7-Dodecenol and Benzyl alcohol were confirmed in *Spirogyra crassa* [21]. It has a role as a plant metabolite. The saturated long chain alcohols are part of chemical intermediates in surfactants preparation and are used in the synthesis of ingredient in personal care and home care products, pharmaceutical formulations, and agrochemicals [5].

Fifty-six different methyl ester compounds were recognized in the various fractions of the three freshwater green macroalgae, (17 in *Chara vulgaris*, 16 in *Cladophora glomerata* and 23 in *Spirogyra crassa*). Out of them, fifty-three compounds were derived from fatty acids and two-ethyl ester were derived from the docosanoic acid, ethyl ester and stearic acid as Octadecanoic acid, ethyl ester while one was derived as hexadecanoic acid, butyl ester. The docosanoic acid, ethyl ester and Octadecanoic acid,

ethyl ester were identified only on the ethyl acetate fraction. The only butyl ester derived was hexadecanoic acid, butyl ester in n-butanol fraction in *Spirogyra crassa*. The total number of ester compounds represented about 50.62% in *Chara vulgaris*, 57.09% in *Cladophora glomerata* and 5.40% in *Spirogyra* by peak percentage in all three fractions. The palmitic acid esters were the major esters in different extracts of these three freshwater green macroalgae followed by stearic acid present in n-hexane, ethyl acetate and n-butanol fraction. The ethyl ester of stearic acid has the capacity to affect the cell cycle and initiates apoptosis in HepG2 cells, further it is a biomarker of excessive alcohol consumption that can be identified in addicted person hair [28]. The esters of fatty acids also acts as allelopathic agents that control the growth of harmful algae and cyanobacterial [29]. Through these phycochemical agents, these macroalgae check and control their competitors in water bodies.

Conclusions

The phycochemical screening through GC-MS analysis of the selected freshwater green macroalgae showed important compounds like ketones, terpenes, esters of fatty acids, fatty alcohols, hydrocarbons, alkaloids. The nature of extracting solvent affect the extracted molecules, especially alkaloids, terpenes and ketones. Amphetamine, phenylpropanolamine (PPA) were detected for the first time, in abundance in these green macroalgae. Several compounds are of highly important due to their medicinal properties. The detection of many antioxidants and PUFA in *Spirogyra crassa* revealed that it could be used as a source of nutraceuticals. In addition, the high content of hydrocarbons and fatty acids make these freshwater green macroalgae as a useful biomass for biodiesel production. The ubiquitous nature of these green macroalgae and the presence of useful compounds in it, make them a suitable and sustainable biomass for future.

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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Figures

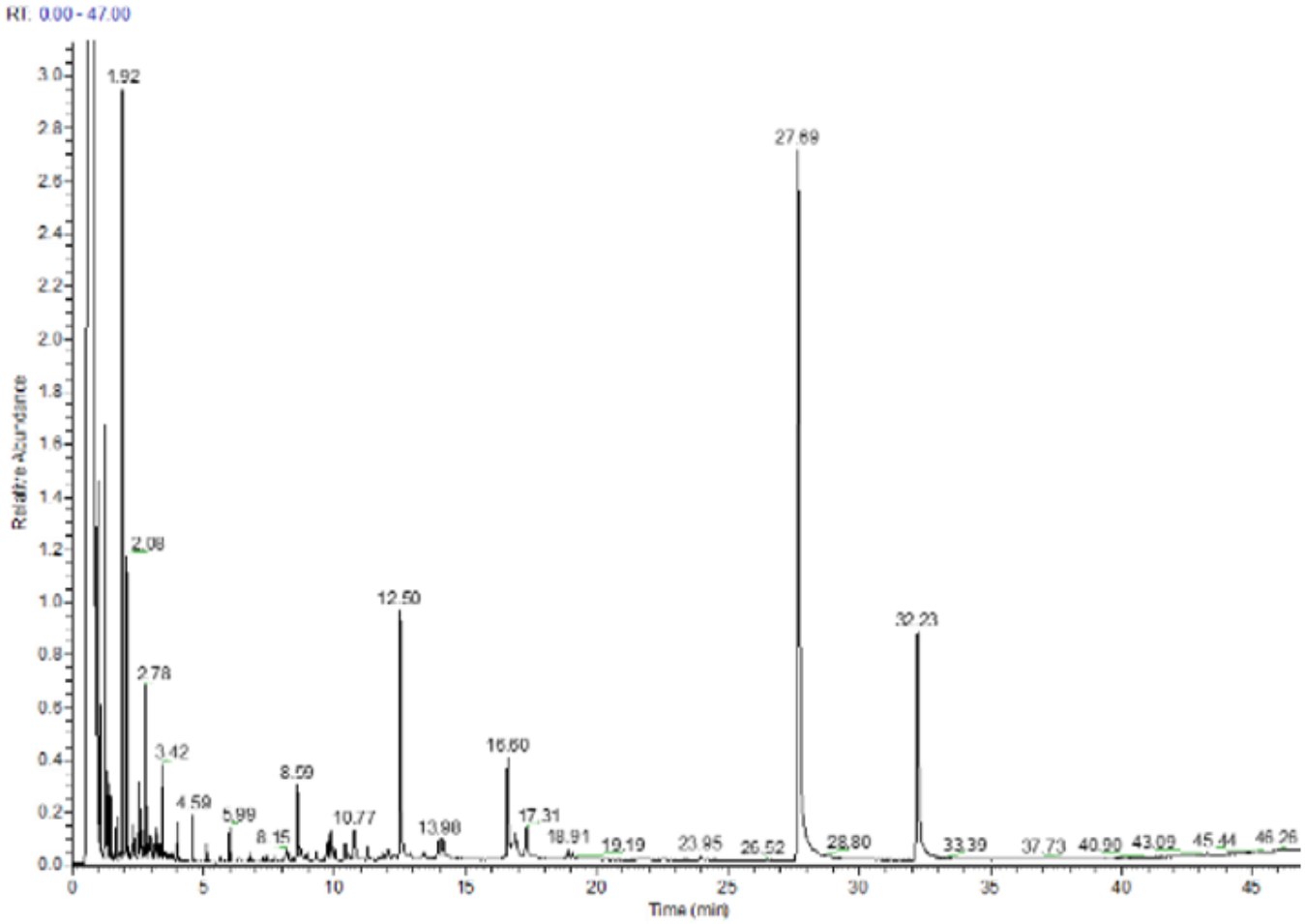

Figure 1

Chromatograms of n-hexane extract of *Chara vulgaris*.

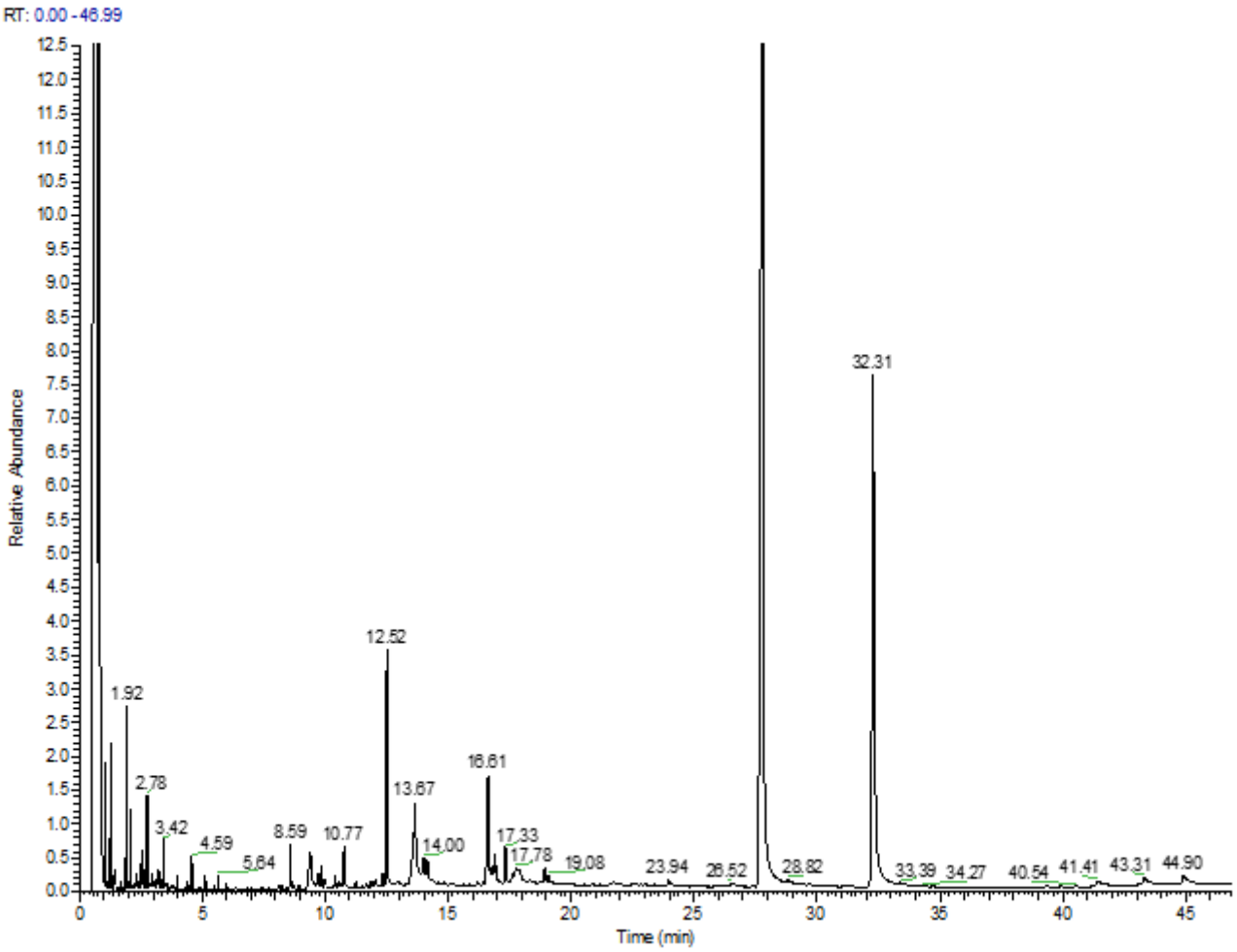

Figure 2

Chromatogram of n-hexane portion of *C. glomerata*.

Figure 3

GC-MS Chromatogram of n-hexane portion *Spirogyra crassa*.

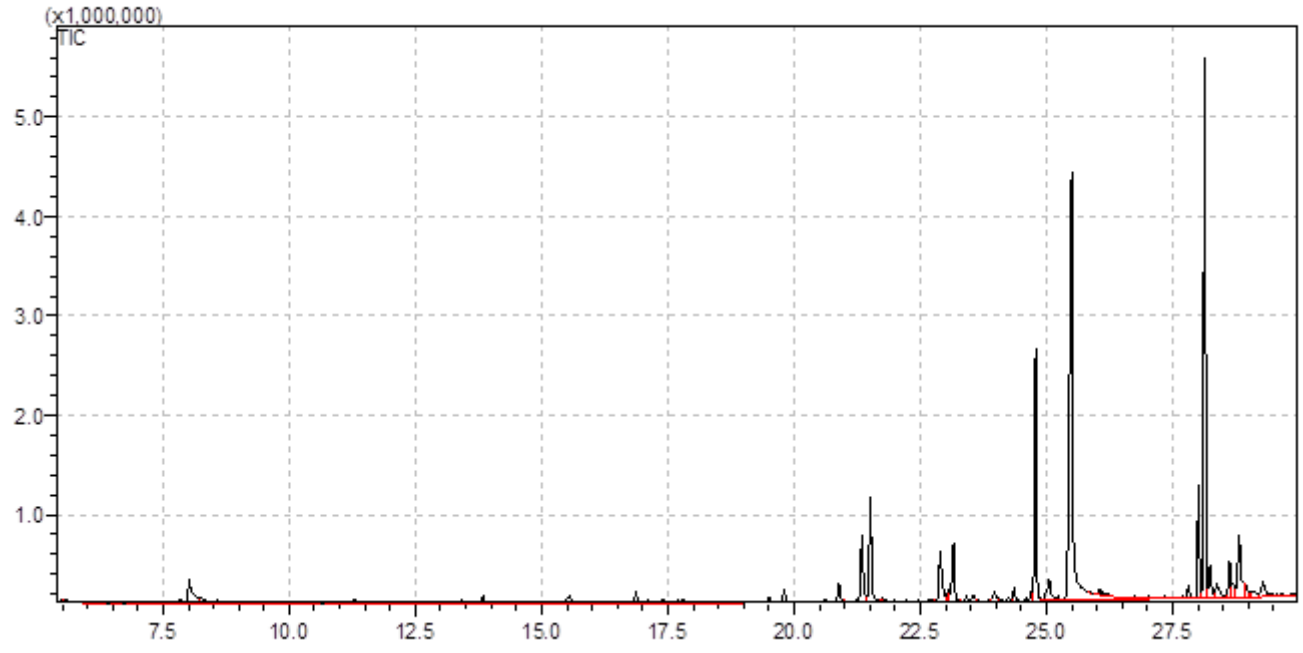

Figure 4

Chromatogram of ethyl acetate portion of *C. vulgaris*.

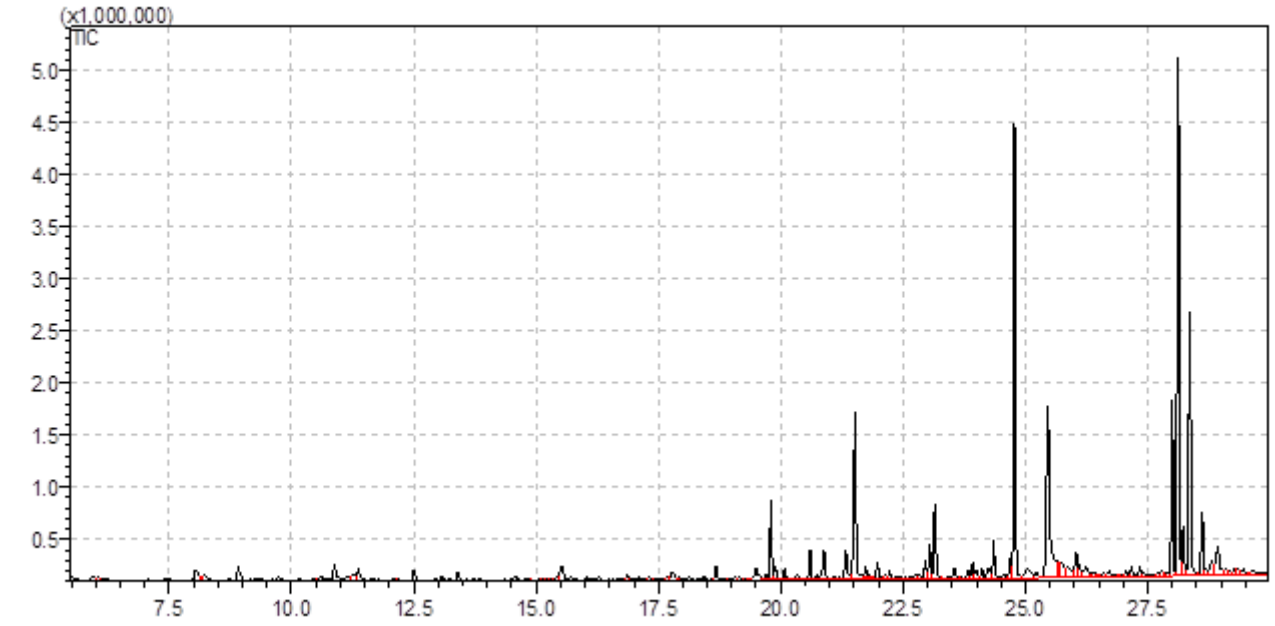

Figure 5

GC-MS Chromatogram of ethylacetate fraction of *C. glomerata*.

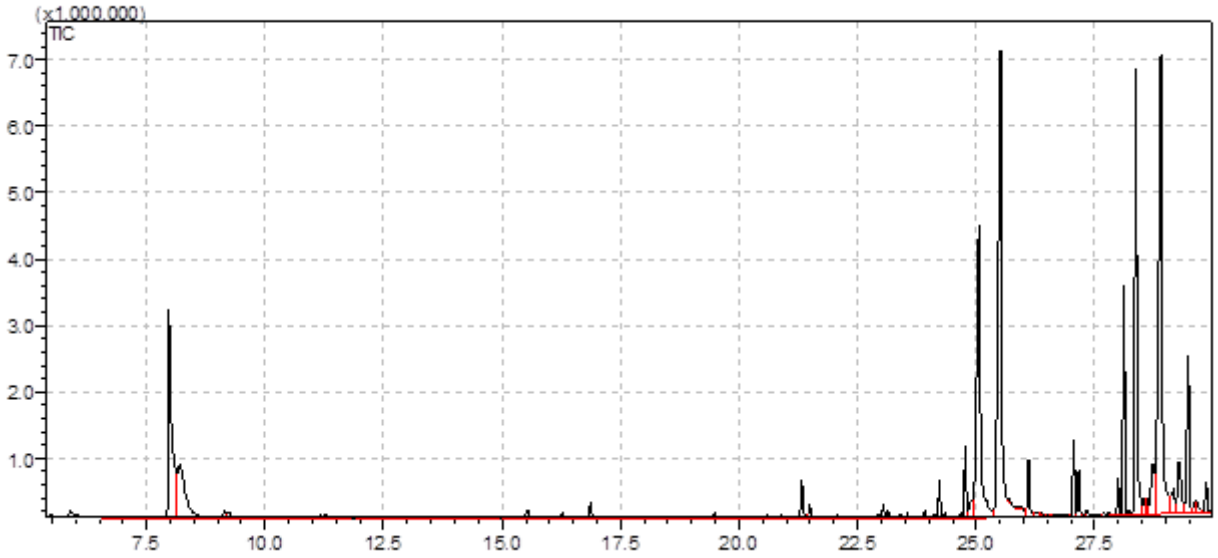

Figure 6

Chromatogram of ethyl acetate portion of the extract from of *S crassa*.

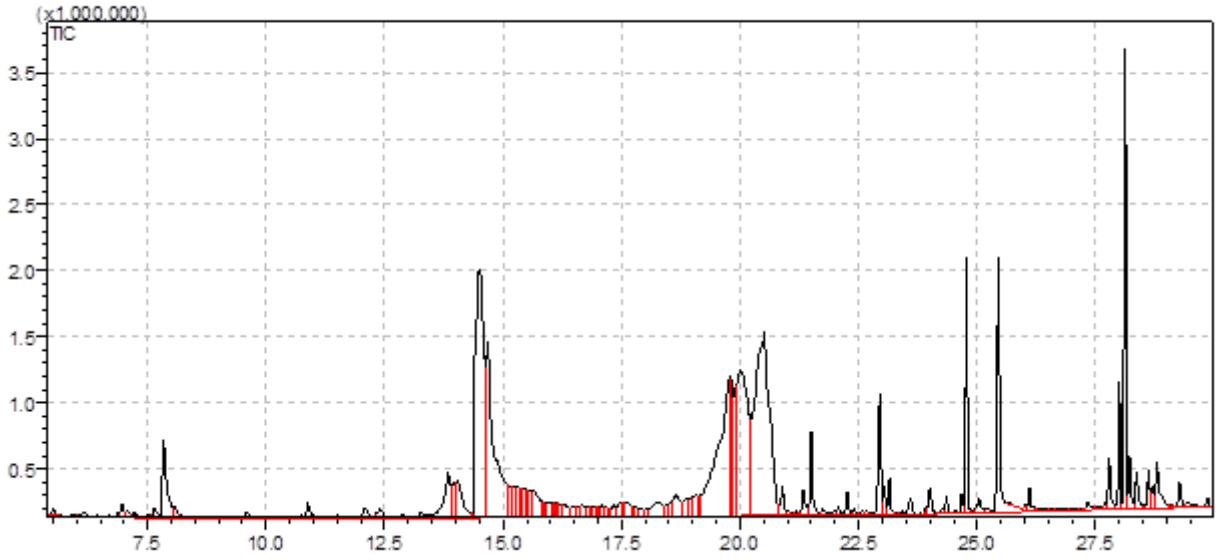

Figure 7

Chromatogram of n-butanol portion of *C vulgaris*.

Figure 8

Chromatogram of n-butanol portion of *C. glomerata*.

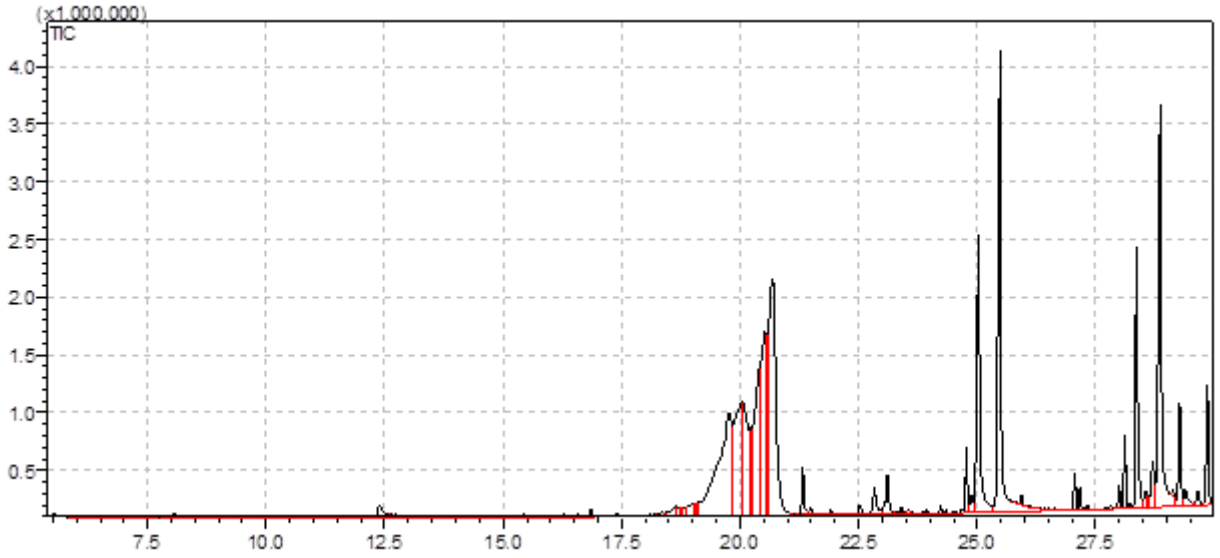

Figure 9

GC-MS chromatogram of the n-butanol fraction of *S. crassa*.