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Chemical composition and antibacterial activity of extracts from freshwater green algae, *Cladophora glomerata* Kützinger and *Microspora floccosa* (Vaucher) Thuret

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ABSTRACT

Freshwater macroalgae, *Cladophora glomerata* Kützinger and *Microspora floccosa* (Vaucher) Thuret, harvested from Nan River in northern Thailand, were extracted with hexane, ethyl acetate, methanol and hot water. The extracts were screened for antibacterial activities. Hexane and ethyl acetate extracts of both algae showed the activities against *Bacillus cereus* and *Vibrio parahaemolyticus*. The extracts were further separated using column chromatography and chemically characterized by GC-MS in order to be tentatively identify the compounds responsible for such activities. The main compositions were fatty acid and other organic compounds, in which have not been reported in these algae. These results indicate that extracts of *C. glomerata* and *M. floccosa* exhibited appreciable antimicrobial activity and could be a source of valuable bioactive materials for health products.

Key words: *Cladophora glomerata*, *Microspora floccosa*, GC-MS, antibacterial activity

Introduction

Traditionally, macroalgae are classified according to chemical and morphological characteristics with special relevance to the presence of specific pigments, which determine the inheritance to one of the three algal divisions: green algae (Chlorophyta), brown algae (Phaeophyta) and red algae (Rhodophyta). Brown algae are the largest type of algae. The brown or yellow-brown colour is due to fucoxanthin. Red algae often have brilliant colour due to phycoerythrin and phycocyanin, which are dominant over the other pigments, as chlorophyll-a, β -carotene and a number of xanthophylls. Green algae contain chlorophyll-a and chlorophyll-b in the same proportion as in higher plants (Domínguez, 2013). Nevertheless, there are few reports of which concerning the activities of macroalgae, for examples, *Sargassum horneri* and *Ecklonia kurome*, produced polyphenols, fucoidans and phlorotannins with antiviral, antibacterial and antioxidative activity (Hoshino et al., 1998; Heo et al., 2005; Kuda et al., 2007). β -Carotene as a vitamin-A precursor from *Dunaliella salina* has already reached

large-scale production. (Pulz & Gross, 2004). Astaxanthin, a pigment component from fresh-water green algae, *Haematococcus pluvialis*, was shown its ability to accumulate under environmental stress. *H. pluvialis* extraction was shown with antioxidant and antimicrobial activity. (Rodríguez-Meizoso et al., 2010)

In northern Thailand, two species of freshwater macroalgae, *C. glomerata* and *M. floccosa*, which belong to the Division Chlorophyta, have been used as a food source and also believed to have many important health benefits such as rejuvenation, induction of appetite and expediting of recovery from many common maladies (Fahprathanchai et al., 2006). According to many advantages of these two algae, but only some few investigations on the chemical composition of *Cladophora* and *Microspora* species have been reported. Diphenyl ethers 2-(2',4'-dibromophenoxy)-4,6-dibromoanisole from the extract of *C. fascicularis*, could inhibit the growth of *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* (Kuniyoshi et al., 1985). Vanillic acid derivatives was isolated from *C. socialis* showed protein tyrosine phosphatase 1B inhibitor activity (Feng et al., 2007).

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Six cycloartane triterpenes were isolated from ethanol extract of *C. fascicularis* (Huang *et al.*, 2006). Eight sterols were isolated from *C. vagabunda* for the chemotaxonomic classification purpose (Aknin *et al.*, 1992). Ten sterols, ursolic acid, hexahydrofarnesylacetone, dihydroactinidiolide, benzyl alcohol, myrtenol and 2-nonanone were also identified in this alga (Elenkov *et al.*, 1995). β -alaninebetaine has been isolated from *C. prolifera*, *C. crispula* and *C. rupestris*. (Blunden *et al.*, 1988). Glycine betaine has been isolated from *C. rupestris* (Blunden *et al.*, 1981). Sulphated heteropolysaccharide has been isolated from *C. socialis* (Sri Ramana & Venkata Rao, 1991). Organic solvent and hot water extracts of both macroalgae were screened for antioxidant and anticancer activities using DPPH free radical scavenging assay and inhibition of proliferation of the KB human oral cancer cell lines respectively (Laungsuwon & Chulalaksananukul, 2013).

In this research, our goal was to study the chemical composition of extracts from fresh water green algae *C. glomerata* and *M. floccosa*, and to determine their antibacterial activity in order to find a potential natural source of bioactive compounds, agrochemical compounds, food supplements and biomedical uses.

Materials and Methods

Raw material, collection and identification

Cladophora glomerata (Figure 1) and *Microspora floccosa* (Figure 2) were collected from Nan Province, Thailand during peak biomass in December 2009 and 2010 when the algae were at their peak biomass (0-2 m in depth). Collected-location are shown in Table 1. Freshly collected algae were washed thoroughly in water to remove epiphytes, small invertebrates and extraneous matter. The samples were separated into two portions: one was used for morphological identification, in comparison with the algae identification book (Bellinger & Sigee, 2010; Barsanti & Gualtieri, 2014), and the other was freeze-dried.

Preparation of extracts

A 100-g portion of each freeze-dried macroalgal sample was extracted successively with 600 mL each of hexane, ethyl acetate and methanol at room temperature. Each extract was next clarified by centrifugation and re-extracted twice with the same solvent. The supernatants were later pooled and filtered. The solvent was then removed from the filtrate by rotary evaporation, and the dry crude extracts were kept at

25°C and protected from light in a desiccator under an atmosphere of nitrogen gas until use.

Table 1. Collected-location of *C. glomerata* and *M. floccosa*

Algae	Address	Location
<i>C. glomerata</i>	Nan River, Pua District	19°11'28.3"N 100°49'50.6"E
<i>M. floccosa</i>	Nan River, Tha Wang Pha District	19°06'59.8"N 100°48'12.6"E

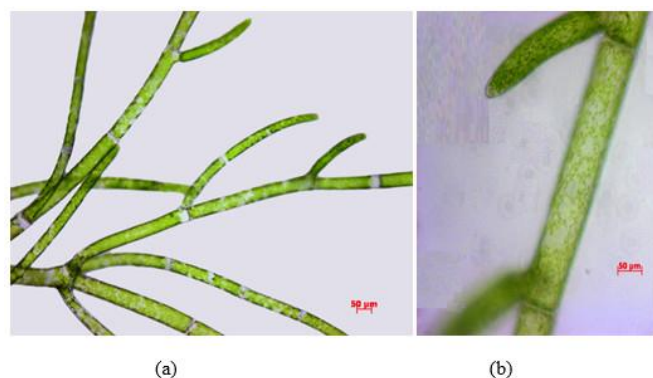


Figure 1. *Cladophora glomerata* Kützinger (a) The branched filament (b) Chloroplast net-like.

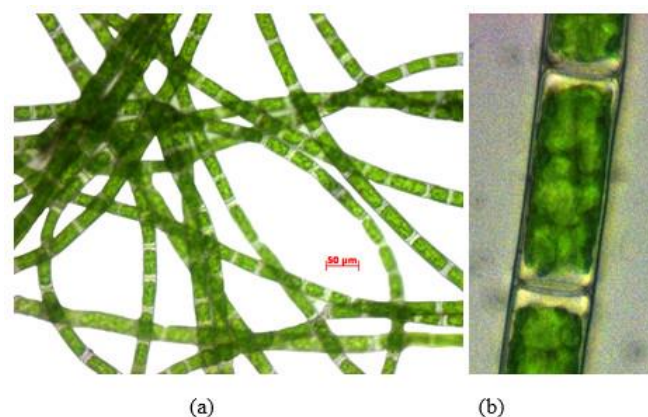


Figure 2. *Microspora floccosa* (Vaucher) Thuret (a) Unbranched filament (b) Two overlapping halves break into H-pieces cell walls.

Another 100 g sample of each freeze-dried alga was extracted with boiling deionized water for 1 h, and the water was removed by lyophilization. The resulting hot water extracts (HWE) were kept at 25°C and protected from light in a desiccator under an atmosphere of nitrogen gas until use.

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Antibacterial determination

Bacterial strains: *Bacillus cereus* TISTR 687 (ATCC 11778) was obtained from Microbiological Resources Centre, Thailand Institute of Scientific and Technological Research (TISTR). *Staphylococcus aureus* ATCC 25923, *Salmonella* Typhimurium ATCC 14028, *Escherichia coli* ATCC 25922 and *Vibrio parahaemolyticus* ATCC 17802 was procured from American Type Culture Collection (ATCC).

Prepare of inoculum by suspending the organism in two ml of sterile saline. Adjust the turbidity of this suspension to a 0.5 McFarland standard, before inoculation of the Mueller-Hinton (MH) agar plates.

Growth inhibition of macroalgae extracts was assessed using the disc diffusion test following CLSI publication, Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard - Ninth Edition. Briefly, 6-mm filter paper disk impregnated with a known concentration of crude extract (200 µg/ml), positive control (Chloramphenicol 50 µg/ml), and negative control (solvent impregnated disc) was allowed to air dry and placed equidistantly onto the surface of the pathogen seeded on MH agar. The plates were kept in an inverted position and incubated at 35±2°C for 24 h. The growth inhibition was measured as the diameter of the zone sizes to the nearest millimeter using a ruler or caliper; include the diameter of the disk in the measurement. The experiment was carried out in triplicate. Experimental data represent mean ± SD of each sample.

Separation of the extracts

Each chemical extraction (hexane, ethyl acetate and hot water extracted) were analysed by Thin-layer chromatography (TLC) with suitable mobile phase. TLC was carried out on a silica gel F254 coated on an aluminium sheet (Merck). TLC chromatograms were detected under UV light at wavelengths 254 and 356 nm and by heating.

All the extracts obtained were separated by column chromatography using silica gel 60 (Merck code no. 7734) or/and Sephadex LH-20 (GE Healthcare code no. 17-0090-01) as packing materials. Collect each similar fraction by TLC. The combined fractions were further purified by repeated chromatography or other purified methods as necessary. Repeating preparation and isolating of extracts have performed to confirm the process.

Chemical composition of the extracts

Gas chromatography - mass spectrometry (GC-MS) analysis was carried out in an Agilent gas chromatography

N7890A fitted with a HP-5MS fused silica column (Agilent 19091S-431, 5% phenyl methyl polysiloxane, 15 m x 0.25 mm, film thickness 0.25 µm), interfaced with an Agilent 7000 with triple quadrupole detector. Oven temperature program was 40°C (hold 2 min) to 150°C at 5°C min⁻¹ and then to 300°C at 15°C min⁻¹. Injector temperature was kept at 280°C. Helium was used as a carrier gas and was adjusted to column velocity flow of 2.0 ml/min⁻¹. Split ratio was 20:1, whereas split flow of 10 ml/min⁻¹, mass range was 50 to 500. 1 µl of sample (dissolved in hexane or ethyl acetate 100% v/v) was injected into the system. Identification of the components was achieved based on retention time and mass spectral matching with GC/MSD ChemStation (G1701DA), Deconvolution Reporting Software (G1716AA), Library searching software NIST MS Search (version 2.0d), Automated Mass Spectral Deconvolution and Identification Software (AMDIS_32), and NIST mass spectral library (G1036A)

Results**Antibacterial determination of *C. glomerata* and *M. floccosa* extracts**

Different solvent polarities, including hexane, ethyl acetate, methanol and hot water, used for extract the bioactive compounds from *C. glomerata* and *M. floccosa*, were screened for antibacterial activity. The results of the antibacterial screening determination are summarized in Table 2 and Table 3. Suggesting its potential for development as a probiotic feed, ethyl acetate extracts of both algae, showed an activity against *Vibrio parahaemolyticus* with the inhibition zones of 11.5±0.7 mm and 8.7±0.8 mm, respectively. Hexane extract of *C. glomerata* showed mild activity against *Staphylococcus aureus* with the inhibition zones of 8.6±0.6 mm, and hexane extract of *M. floccosa* also showed activity against *Bacillus cereus* with the inhibition zones of 8.9±0.9 mm. Only methanol extract of *C. glomerata* possessed activity against *Vibrio parahaemolyticus*. All of algae extracts showed no activity against *Salmonella typhimurium* and *Escherichia coli*.

Separation of the extracts

Isolation of hexane extract from *C. glomerata* (Figure 3): 2.1 g of the hexane extract from *C. glomerata* was subjected to silica gel chromatography eluted with hexane-ethyl acetate (2:1) to afford ten fractions (1-10). Fractions 2-3 (167.8 mg) were rechromatographed over silica gel eluted with hexane-

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ethyl acetate (3:1) to afford six fractions (1'-6'). Fraction 2' (58.3 mg) and fraction 6' (71.0 mg) were further separately subjected to Sephadex LH-20 eluted with chloroform-methanol (1:1) to yield H1 (12.2 mg) and H2 (25.1 mg).

Isolation of hexane extract from *M. floccosa* (Figure 4): 2.1 g of the hexane extract from *M. floccosa* was subjected to silica gel column chromatography eluted with hexane-ethyl acetate (3:1) to afford eighth fractions (1-8). Fraction 3-4 (167.8 mg) were rechromatographed over silica gel eluted with hexane-ethyl acetate (4:1) to afford ten fractions (1'-10'). Fractions 4'-6' (71.0 mg) were further separately

subjected to Sephadex LH-20 column eluted with chloroform-methanol (1:1) to yield H3 (25.1 mg).

Isolation of ethyl acetate extract from *C. glomerata* (Figure 5): 2.0 g of the ethyl acetate extract from *C. glomerata* was subjected to silica gel column chromatography eluted with chloroform-methanol (5:1) to afford twelve fractions (1-12). Fraction 6 (335.0 mg) was rechromatographed over silica gel eluted with chloroform-methanol (6:1) to afford six fractions (1'-6'). Fraction 3' (125.3 mg) was further subjected to Sephadex LH-20 column eluted with methanol to yield E1 (32.2 mg).

Table 2. Antibacterial activity of *C. glomerata* extracts

Extracts	Inhibition zone (mm)				
	hexane	ethyl acetate	methanol	hot water	chloramphenicol
Gram-positive bacteria					
<i>Bacillus cereus</i> ATCC 11778	-	-	-	-	15.8±0.3
<i>Staphylococcus aureus</i> ATCC 25923	8.6±0.6	-	-	-	14.5±0.4
Gram-negative bacteria					
<i>Escherichia coli</i> ATCC 25922	-	-	-	-	11.8±0.4
<i>Salmonella typhimurium</i> ATCC 14028	-	-	-	-	13.5±0.3
<i>Vibrio parahaemolyticus</i> ATCC 17802	-	11.5±0.7	9.7±0.5	-	12.6±0.5

Note: Mean±SD (n=3), '-' : no activity

Table 3. Antibacterial activity of *M. floccosa* extracts

Extracts	Inhibition zone (mm)				
	hexane	ethyl acetate	methanol	hot water	chloramphenicol
Gram-positive bacteria					
<i>Bacillus cereus</i> ATCC 11778	8.9±0.9	-	-	-	15.8±0.3
<i>Staphylococcus aureus</i> ATCC 25923	-	-	-	-	14.5±0.4
Gram-negative bacteria					
<i>Escherichia coli</i> ATCC 25922	-	-	-	-	11.8±0.4
<i>Salmonella typhimurium</i> ATCC 14028	-	-	-	-	13.5±0.3
<i>Vibrio parahaemolyticus</i> ATCC 17802	-	8.7±0.8	-	-	12.6±0.5

Note: Mean±SD (n=3), '-' : no activity

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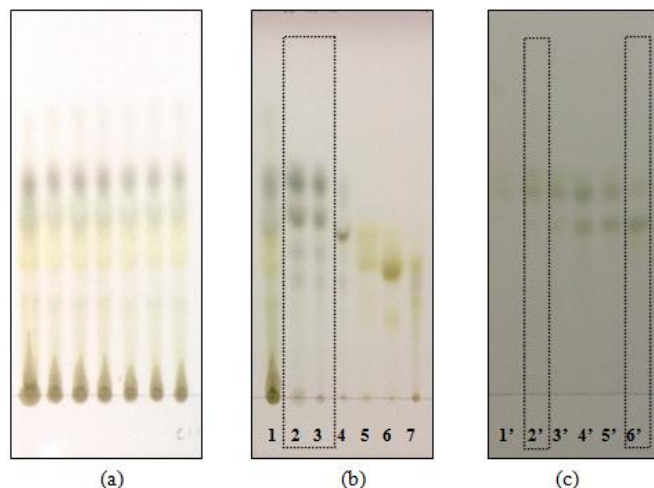


Figure 3. TLC from *C. glomerata* (a) hexane extract (b) fraction 2-3 (c) fraction 2' and 6'

Isolation of ethyl acetate extract from *M. floccosa* (Figure 6): 2.0 g of the ethyl acetate extract from *M. floccosa* was subjected to silica gel column chromatography eluted with chloroform-methanol (5:1) to afford ten fractions (1-10). Fractions 8-10 (222.4 mg) were rechromatographed over silica gel eluted with chloroform-methanol (5:1) to afford eighth fractions (1'-8'). Fractions 6'-7' (89.1 mg) were further subjected to Sephadex LH-20 column eluted with methanol to yield E2 (24.7 mg).

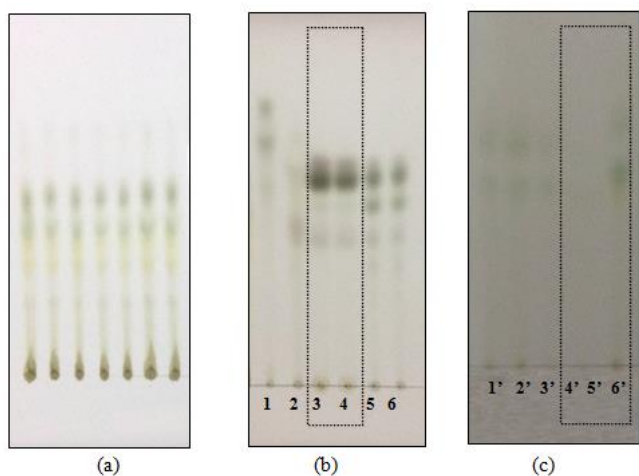


Figure 4. TLC from *M. floccosa* (a) hexane extract (b) fraction 3-4 (c) fraction 4'-6'

Chemical composition of the extracts

At present, analysis by Gas chromatography-mass spectrometry (GC-MS) is essential for the identification of

natural organic compounds. *C. glomerata* and *M. floccosa* extracts are chemically characterized in order to determine the compounds responsible for the biological activity observed using GC-MS techniques. Different natural antimicrobial compounds have been described in algae belonging to a wide range of chemical classes, including indoles, terpenes, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons (Rodríguez-Meizoso et al., 2010). Thus, GC-MS methods were used to analyze both, fatty acids and volatile compounds, in separated fraction (H1,H2,H3,E1 and E2) of hexane and ethyl acetate extracts that showed antibacterial activity from the two studied algae.

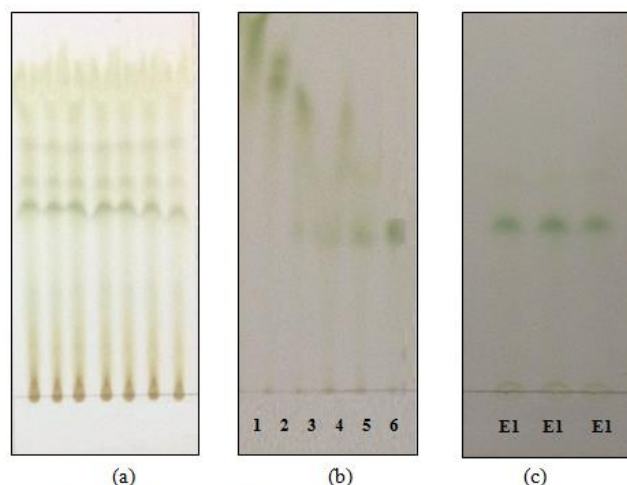


Figure 5. TLC from *C. glomerata* (a) ethyl acetate extract (b) fraction 1-6 (c) E1

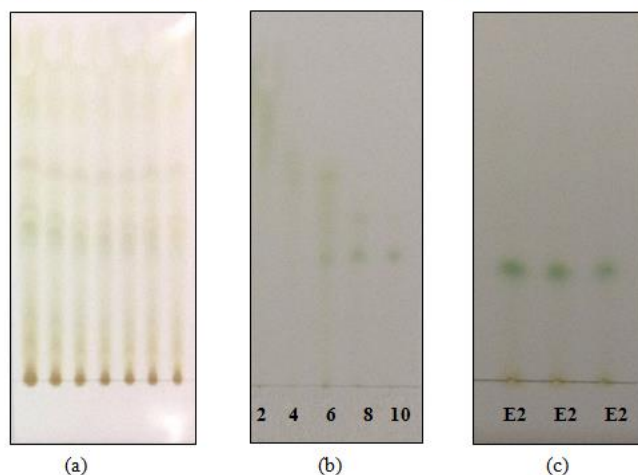


Figure 6. TLC from *M. floccosa* (a) ethyl acetate extract (b) fraction 1-10 (c) E2

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As can be seen in Tables 4-8, several volatile compounds were identified in extracts from *C. glomerata* and *M. floccosa*, mainly fatty acids, alkanes, phenols and compounds such as imidazole, 2-amino-5-[(2-carboxy)vinyl]-, 2,4-di-tert-butylphenol and dihydroactinidiolide. Fatty acids such as

lauric, palmitic, linolenic, linoleic, oleic, stearic and myristic have been already proposed to have certain antimicrobial activity (Agoramoorthy et al., 2007). The activity found in these algae may be related with the amount of fatty acids existing in the extracts.

Table 4. GC-MS analysis of major compounds of H1

RT (min)	Compound	Peak area (%)	Molecular formula	MW	Retention index (IU)
36.558	Methyl tetradecanoate (Myristic acid, methyl ester)	5.42	C ₁₅ H ₃₀ O ₂	242.40	1680
39.421	Ppropionic acid, 3-(1- hydroxy-2-isopropyl -5-methylcyclohexyl)	11.16	C ₁₃ H ₂₀ O ₃	224.30	1760

Table 5. GC-MS analysis of major compounds of H2

RT (min)	Compound	Peak area (%)	Molecular formula	MW	Retention index (IU)
36.559	Methyl tetradecanoate (Myristic acid, methyl ester)	18.67	C ₁₅ H ₃₀ O ₂	242.40	1680
39.423	Ppropionic acid, 3-(1- hydroxy-2-isopropyl -5-methylcyclohexyl)	2.23	C ₁₃ H ₂₀ O ₃	224.30	1760

Table 6. GC-MS analysis of major compounds of H3

RT (min)	Compound	Peak area (%)	Molecular formula	MW	Retention index (IU)
50.281	Methyl 4,7,10,13- hexadecatetraenoate	6.76	C ₁₇ H ₂₆ O ₂	262.39	1910
51.553	Dodecanoic acid, methyl ester (Lauric acid, methyl ester)	7.25	C ₁₃ H ₂₆ O ₂	214.34	1481
54.068	9,12,15-Octadecatrienoic acid, (z,z,z)- (Linolenic acid)	9.88	C ₁₈ H ₃₀ O ₂	278.43	2191
50.281	Methyl 4,7,10,13-hexadecatetraenoate	6.76	C ₁₇ H ₂₆ O ₂	262.39	1910

Table 7. GC-MS analysis of major compounds of E1

RT (min)	Compound	Peak area (%)	Molecular formula	MW	Retention index (IU)
20.866	Imidazole, 2-amino-5-[(2-carboxy)vinyl]	6.52	C ₆ H ₇ N ₃ O ₂	153.14	1693
26.112	Phenol, 2,4-bis(1,1-dimethylethyl)- (2,4-di-tert-butylphenol)	12.41	C ₁₄ H ₂₂ O	206.32	1555
26.955	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro -4,4,7a-trimethyl-, (R)-(dihydroactinidiolide)	4.45	C ₁₁ H ₁₆ O ₂	180.24	1426

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Table 8. GC-MS analysis of major compounds of E2

RT (min)	Compound	Peak area (%)	Molecular formula	MW	Retention index (IU)
4.093	Propanoic acid, 2-methyl-, methyl ester (Isobutyric acid, methyl ester)	1.14	C ₅ H ₁₀ O ₂	102.13	621
4.230	Butane, 1-ethoxy-	2.39	C ₆ H ₁₄ O	102.17	694

Conclusion

The two freshwater green algae, *C. glomerata* and *M. floccosa*, have been identified as a rich and renewable source of biologically active compounds that may be useful as therapeutic agents with antioxidant, anticancer (Laungsuwon & Chulalaksananukul, 2013) and antibacterial activity. The main identified compounds were fatty acid: myristic acid, methyl ester [1]; ppropionic acid [2], 3-(1-hydroxy-2-isopropyl-5-methylcyclohexyl) [3]; dodecanoic acid, methyl ester [4]; 9,12,15-Octadecatrienoic acid, (z,z,z)-[5]; propanoic acid, 2-methyl-, methyl ester [6] and the other compounds such as imidazole, 2-amino-5-[(2-carboxy)vinyl]-[7]; 2,4-di-tert-butylphenol [8]; dihydroactinidiolide [9] and butane, 1-ethoxy- [10].

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