ORIGINAL ARTICLE

RECORDS OF PHARMACEUTICAL AND BIOMEDICAL SCIENCES

Gas Chromatography-Mass Spectrometry Analysis of Marine Seagrass *Thalassodendron ciliatum* Collected from Red Sea

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Abstract

Marine organisms are considered as a treasure for discovery of bioactive metabolites. Seagrasses provide food and habitat for other marine organisms. They are widely distributed in tropical and subtropical regions. Seagrass *Thalassodendron ciliatum* (Forsk.) den Hartog is a very common seagrass species in the Red Sea. Several studies have proved its antioxidant, anti-inflammatory and antimicrobial potentials. Gas Chromatography-Mass spectrometry Analysis was performed for identification of existed phytochemicals. Herein, it is the first reported study explaining the lipoidal matter of *n*-hexane fraction of *T. ciliatum* using GC-MS technique. The obtained data revealed presence of saturated and unsaturated- long chain- fatty acids; tetradecanoic acid, eicosanoic acid, 9,12-hexadecadienoic acid and 8,11,14-eicosatrienoic acid, in addition to other volatile compounds; 1-heneicosanol, 2,6-bis (1,1-dimethylethyl) phenol and 1-tridecanol. These compounds were previously assessed for their antibacterial, antifungal, antimicrobial and anti-inflammatory bioactivities. So, the previously reported antioxidant, anti-inflammatory and antimicrobial activities of *Thalassodendron ciliatum* may be attributed to these identified compounds.

Keywords: GC-MS, *Thalassodendron ciliatum*, tetradecanoic acid, 1-heneicosanol, antimicrobial.

1. Introduction

The marine environment (>70% of the planet's surface) possesses unique biological and chemical characters that play a vital role in discovery of many drug leads. Many marine-living organisms are soft bodied and/or sessile. Consequently, they have developed toxic secondary metabolites to defend themselves against predators (Eltamany, 2015). The Red Sea is considered as a precious natural source of bioactive compounds because of its distinctive features; lack of any river drainage, great marine organisms' biodiversity, and seasonal fluctuations of air and water temperatures. According to El- Ezz et al., several terpeniods, alkaloids, sterols and steroidal glycosides were

reported in Red Sea marine organisms besides, other metabolites of diverse chemical classes (El-Ezz et al., 2017). Scientists classified marine organisms into different categories; Bacteria (ex. marine bacteria), Protocists (ex. algae), Plants (ex. sea grass) and Animals (ex. sponges, corals and tunicates). This biodiversity ensures presence of different classes of secondary metabolites that manifested beneficial biological activities. Sea grasses can be found all over the world except in the polar region. In many places, sea grasses cover extensive areas, which are usually called as sea grass beds. *Thalassodendron ciliatum* (Forsk.) den Hartog is commonly known as 'Majani kumbi', it is a very common seagrass species in the Red Sea. *T. ciliatum* is a tropical sea grass which can be classified as sub-tidal and not deep-water bed forming sea grass. Traditional healers prescribed seagrasses as an effective treatment for different ailments (Ibrahim et al., 2013; Mohammed et al., 2019). Some previous studies reported isolation and identification of different phytochemicals like caffeic acid, catechin, asebotin, quercetin-3-O-*β*-D-xylopyranoside, rutin, 6-O-rhamnosyl-(1‴ → 6‴) glucopyranosyl asebogenin, diglyceride ester, 7*β*-hydroxy cholesterol, 7*β*-hydroxysitosterol, stigmasterol glucoside, *β*-sitosterol glucoside and ceramides, as shown in figure 1 (Hamdy et al., 2012; Ibrahim et al., 2013; Mohammed et al., 2014; Abdelhameed et al., 2018). Moreover, *T. ciliatum* (Forsk.) den Hartog showed a significant antioxidant, anti-inflammatory, antiviral and cytotoxic activities. So, further studies are needed for discovery of other natural compounds present in *T. ciliatum.* To the best of our knowledge, few previous studies were reported concerning chemical investigation of *T. ciliatum.* Herein, it is the first reported one explaining the lipoidal matter of *T. ciliatum* using GC-MS technique.

2. Experimental Section

2.1. Marine material and chemicals

Seagrass *Thalassodendron ciliatum* was collected from Sharm El-Sheikh, Egypt. It was taxonomically identified by Prof. Tarek Temraz Marine Science Department, Faculty of Science, Suez Canal University, Ismailia, Egypt. A voucher specimen was deposited in the herbarium section of Pharmacognosy Department, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt under the registration number SAA-41. Solvents used for extraction and fractionation were analytical grade, but subjected to distillation before use. While, solvents of HPLC grade were used for GC-MS analysis.

2.2. Instruments

Rotatory evaporator (Buchi®, G. Switzerland) was used for evaporation, while GC–MS instrument (TRACE GC Ultra Gas Chromatographs; THERMO Scientific Corporation, Waltham, Massachusetts, USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer, Thermo Scientific, San Jose, California, USA) was used for GC-MS analysis.

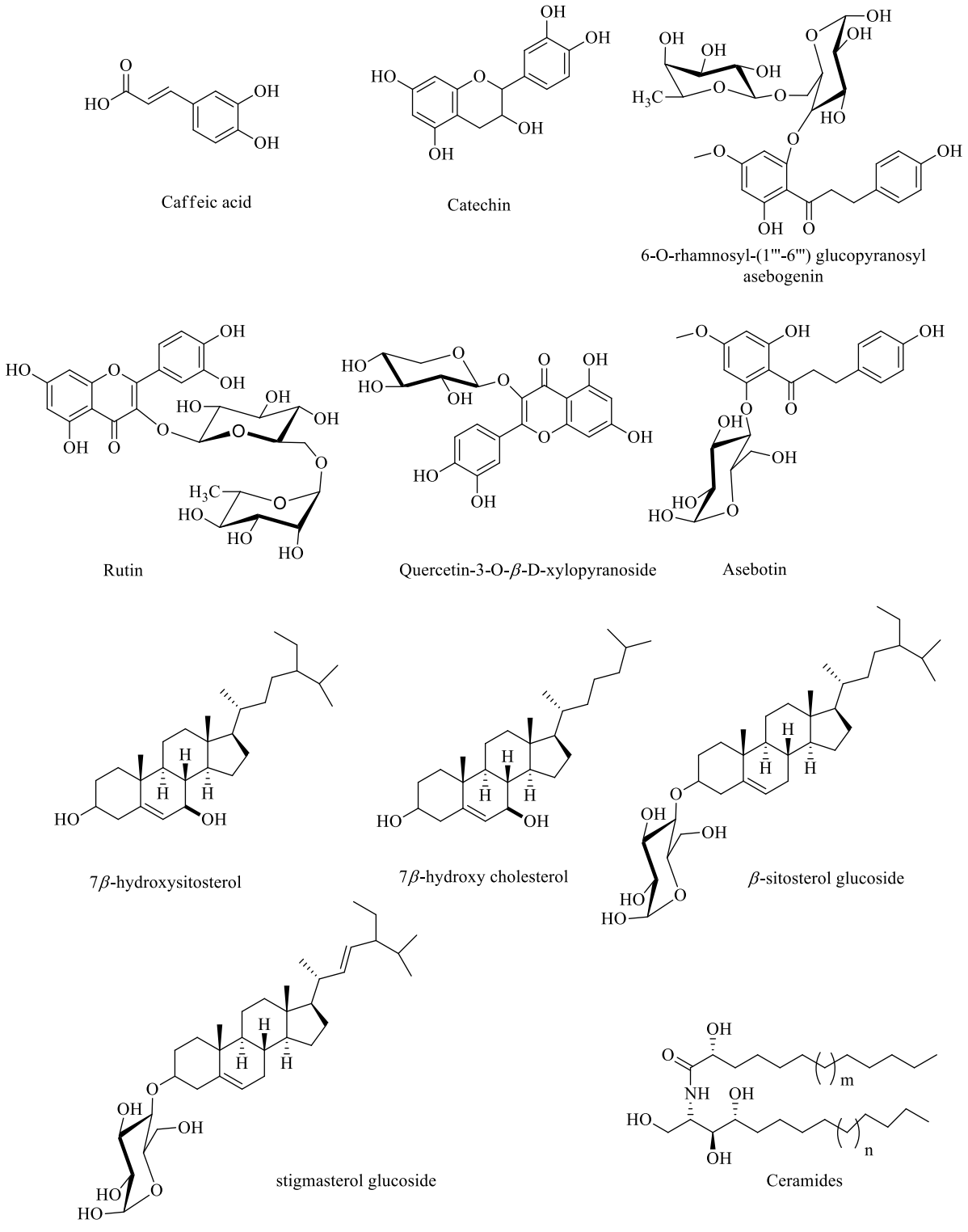
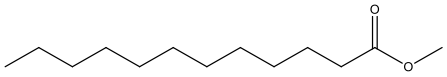
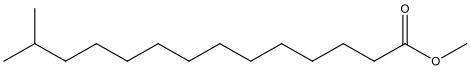
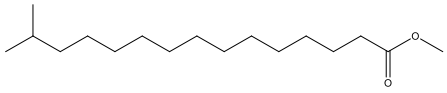
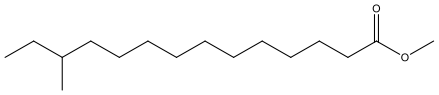
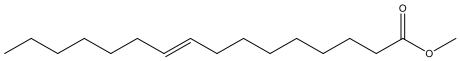
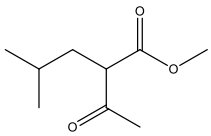
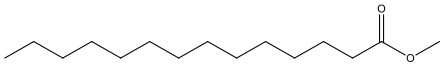
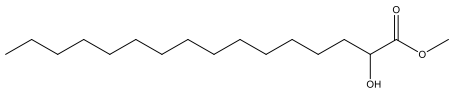
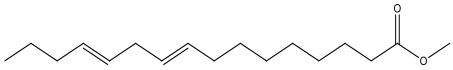
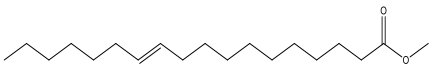
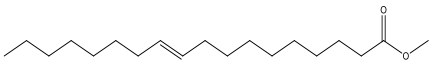
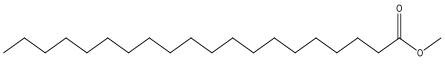
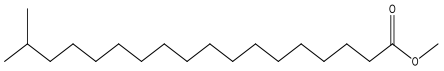
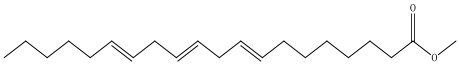
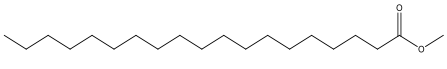
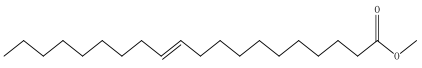
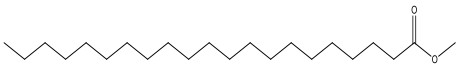
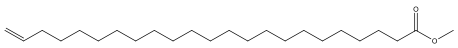
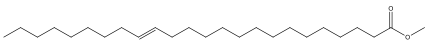
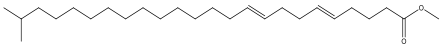
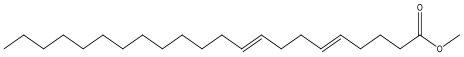


Figure 1: structures of previously isolated compounds from *Thalassodendron ciliatum*

Table 1: Fatty acid methyl esters of *Thalassodendron ciliatum*

	Retention time (min)	Name of compound	Structure	Area%	Molecular formula	Molecular weight
1	4.98	Dodecanoic acid, methyl ester		0.55	$C_{13}H_{26}O_2$	214
2	9.97	Methyl-13-methyltetradecanoate		1.48	$C_{16}H_{32}O_2$	256
3	12.18	Pentadecanoic acid, 14-methyl-, methyl ester		0.72	$C_{17}H_{34}O_2$	270
4	10.79	Tetradecanoic acid, 12-methyl-, methyl ester		6.07	$C_{16}H_{32}O_2$	256
5	12.57	9-Hexadecenoic acid, methyl ester		4.68	$C_{17}H_{32}O_2$	268
6	13.08	Pentanoic acid, 2-acetyl-4-methyl-, methyl ester		21.13	$C_9H_{16}O_3$	172
7	15.27	Tetradecanoic acid, methyl ester		11.77	$C_{15}H_{30}O_2$	242
8	15.74	2-hydroxy- Hexadecanoic acid, methyl ester		0.2	$C_{17}H_{34}O3$	286
9	16.57	9,12-Hexadecadienoic acid, methyl ester		1.14	$C_{17}H_{30}O_2$	266
10	16.88	11-Octadecenoic acid, methyl ester		2.13	$C_{19}H_{36}O_2$	296
11	17.01	10-Octadecenoic acid, methyl ester		5.40	$C_{19}H_{36}O_2$	296
12	17.47	Eicosanoic acid, methyl ester		11.25	$C_{21}H_{42}O_2$	326
13	18.78	Octadecanoic acid, 17-methyl-, methyl ester		2.21	$C_{20}H_{40}O_2$	312
14	19.31	8,11,14-Eicosatrienoic acid, methyl ester		0.4	$C_{21}H_{36}O_2$	320
15	19.56	Nonadecanoic acid, methyl ester		1.41	$C_{20}H_{40}O_2$	312

16	21.24	11-Eicosenoic acid, methyl ester		0.55	$C_{21}H_{40}O_2$	324
17	23.65	Heneicosanoic acid, methyl ester		1.14	$C_{22}H_{44}O_2$	340
18	20.27	22-Tricosenoic acid, methyl ester		0.56	$C_{23}H_{44}O_2$	352
19	29.06	15-Tetracosenoic acid, methyl ester		0.59	$C_{25}H_{48}O_2$	380
20	30.40	Methyl 23-methyl-tetracosa-5,9-dienoate		1.81	$C_{26}H_{48}O_2$	392
21	32.20	Methyl 5,9-docosadienoate		4.92	$C_{23}H_{42}O_2$	350

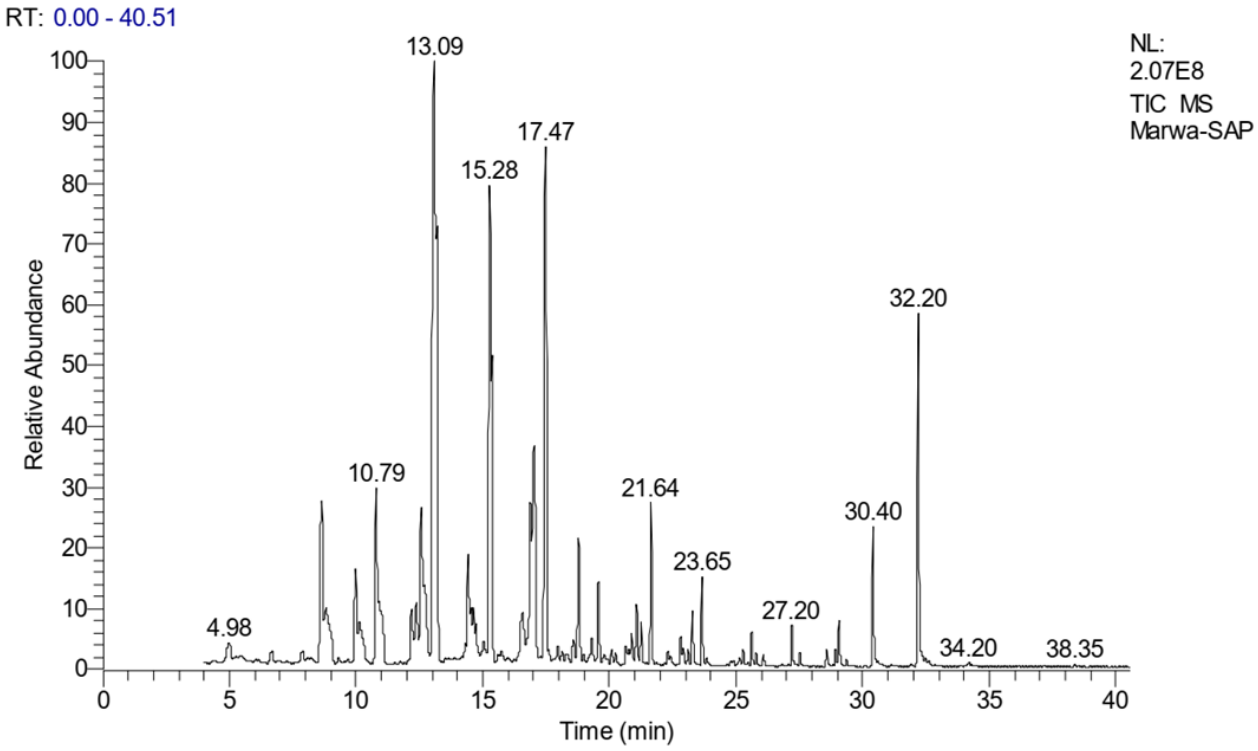


Figure 2: Chromatogram of GC-MS analysis of fatty acid methyl esters of sea grass *Thalassodendron ciliatum*

2.3. Extraction and fractionation of marine material

Thalassodendron ciliatum of 90 g. weight was dried, grounded and soaked in methanol for 3 days at room temperature. This soaking or extraction process was repeated for 3 times to ensure complete extraction of active metabolites. Then, the combined methanol extract was concentrated under vacuum resulting in 47.4 g. residue. The residue was fractionated using open column technique with *n*-hexane, ethyl acetate and methanol gradient elution. Fractions of different polarities were also evaporated under vacuum and weighed. The resulting *n*-hexane fraction of 5.76 g. was subjected for Gas Chromatography-Mass spectrometry [GC-MS] analysis.

2.4. GC-MS analysis of *n*-hexane extract

2.4.1. Preparation of unsaponifiable matter

One gram of *n*- hexane fraction was refluxed with 50 ml of 10% ethanolic potassium hydroxide and 20 ml benzene at 90 ℃ for 24 hours for saponification process. After this, the mixture was evaporated and concentrated to its third volume under vacuum. 100 ml of distilled water were added to the residue and then the aqueous solution was partitioning with diethyl ether (5X100 ml) for several times using a separating funnel till complete extraction of unsaponifiable matter. The collected extracts of diethyl ether were washed with distilled water for several times, then dried over anhydrous sodium sulphate. The residue weighed 0.46 g. of unsaponified matter (Eltamany, 2010).

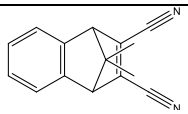
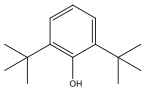
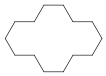
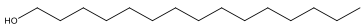
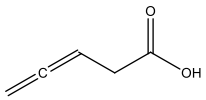
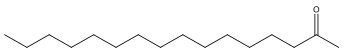
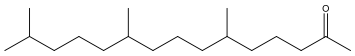
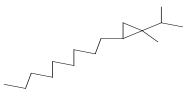
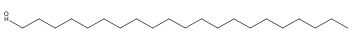
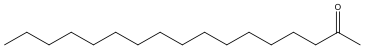
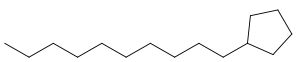
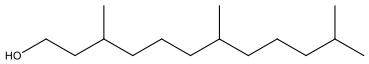
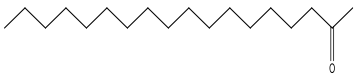
2.4.2. Preparation of fatty acid methyl ester

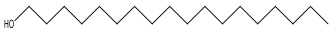
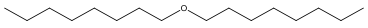
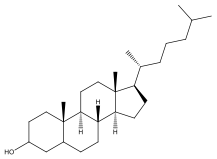
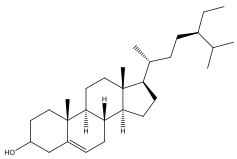
The aqueous alkaline solution left after separation of unsaponified matter was acidified with conc. HCl using litmus paper as indicator for pH. The liberated fatty acids were extracted with ether for several times using a separating funnel till complete extraction. The collected extracts of ether were washed with distilled water for several times till washing is neutral to litmus paper, then dried over anhydrous sodium sulphate. The residue weighed 0.34 g. of free fatty acids. For preparation of fatty acid methyl ester, 0.2 g. of residue was refluxed with 50 ml absolute methanol and 2.5 ml conc. H_2SO_4 at 85 ℃ for 2 hours. After cooling, the mixture was diluted with 100 ml of distilled water and extracted with diethyl ether for several times using a separating funnel till complete extraction of fatty acid methyl esters. The collected extracts of diethyl ether were washed with distilled water for several times till neutral to litmus paper, then dried over anhydrous sodium sulphate (Eltamany, 2010).

2.4.3. GC-MS analysis

Both fatty acid methyl esters and unsaponifiable matter were analyzed using GC-MS technique. The GC–MS system was equipped with a TG-WAX MS column (30 m×0.251 mm daily, 0.1 mm film thickness). Analysis was carried out using helium as a carrier gas at a constant flow rate of 1.0 ml/min. Diluted samples (1:10 chloroform, v/v) of 0.2µl of the mixtures were always injected automatically in the splitless mode. The injector and MS transfer line temperature were set at 280°C. The separation technique for fatty acid methyl esters was carried out using the following temperature program: 150°C for 4 min as an initial temperature

Table 2: Hydrocarbons and their derivatives of *Thalassodendron ciliatum*

	Retention time (min)	Name of compound	Structure	Area%	Molecular formula	Molecular weight
1	22.37	2,3-dicyano-7,7-dimethyl -5,6-benzonorbornadiene		2.36	$C_{15}H_{12}N_2$	220
2	22.52	Phenol, 2,6-bis (1,1-dimethylethyl)		6.32	$C_{14}H_{22}O$	206
3	24.05	1-Hexadecanol		1.0	$C_{16}H_{34}O$	242
4	26.03	Cyclotetradecane		2.71	$C_{14}H_{28}$	196
5	27.39	1-Tridecanol		2.03	$C_{13}H_{28}O$	200
6	28.19	1-Pentadecanol		6.60	$C_{15}H_{32}O$	228
7	28.39	Penta-3,4-dienoic acid		9.49	$C_5H_6O_2$	98
8	28.59	2-Hexadecanone		1.58	$C_{16}H_{32}O$	240
9	29.46	2-Pentadecanone, 6,10,14-trimethyl		1.03	$C_{18}H_{36}O$	268
10	29.63	Cyclopropane, 1-methyl-1-(1-methylethyl)-2-nonyl		1.10	$C_{16}H_{32}$	224
11	30.26	1-Heneicosanol		13.94	$C_{21}H_{44}O$	312
12	30.49	Eicosane		0.7	$C_{20}H_{42}$	282
13	30.61	2-Heptadecanone		3.58	$C_{17}H_{34}O$	254
14	32.17	Cyclopentane, decyl		1.16	$C_{15}H_{30}$	210
15	32.32	1-Dodecanol, 3,7,11-trimethyl		12.44	$C_{21}H_{44}O$	312
16	32.53	Methyl n-hexadecyl ketone		3.21	$C_{18}H_{36}O$	268

17	34.02	1-Octadecanol		2.57	$C_{18}H_{38}O$	270
18	35.88	Octane, 1,1'-oxybis		10.16	$C_{16}H_{34}O$	242
19	39.15	1-Hexacosanol		5.56	$C_{26}H_{54}O$	382
20	42.26	1-Dotriacontanol		1.60	$C_{32}H_{66}O$	466
21	49.99	Cholestan-3-ol		0.43	$C_{27}H_{48}O$	388
22	52.50	Stigmast-5-en-3-ol		0.22	$C_{29}H_{50}O$	414

with an increasing rate of 5.0°C/min till 280°C as a final temperature and held for 4 min. While, the temperature program for unsaponifiable matter was programmed at an initial temperature 50 °C (hold 2 min) to150 °C with an increasing rate of 7 °C /min then to 270 °C with an increasing rate 5 °C /min (hold 2min) then to 310 °C as a final temperature at an increasing rate of 3.5 °C /min (hold 10 min). For GC/MS detection an electron ionization system with ionization energy of 70 eV was used with a spectral range of *m/z* 40–450. Identification of the phytochemical constituents was carried out by comparison of their retention time and fragmentation pattern of mass spectra with those of published data and/or with those of the Wiley 9 and NIST08 mass spectral libraries. The quantification of all the identified components was investigated using a percent relative peak area (Elkhateeb et al., 2019).

3. Results and Discussion

3.1.GC-MS analysis of fatty acid methyl ester

More than 40 fatty acid methyl esters were identified in the saponifiable part of the *n*-hexane fraction of *T. ciliatum* (Table 1). The saturated fatty acid methyl ester derivatives represented 73.5% of total fatty acids in the lipoidal matter of *T. ciliatum* while unsaturated fatty acid constituted 26.5% of total fatty acid content. According to the obtained GC chromatogram (Figure 2), the major fatty acid methyl ester derivatives recorded were; 2-acetyl-4-methyl-pentanoic (21.13 %) tetradecanoic acid (11.77%) and eicosanoic acid (11.25%). Moreover, methyl esters of 12-methyl- tetradecanoic acid, 10-octadecenoic acid, 5,9-docosadienoic acid, 17-methyl-octadecanoic acid, 9-hexadecenoic acid (palmitoleic acid),

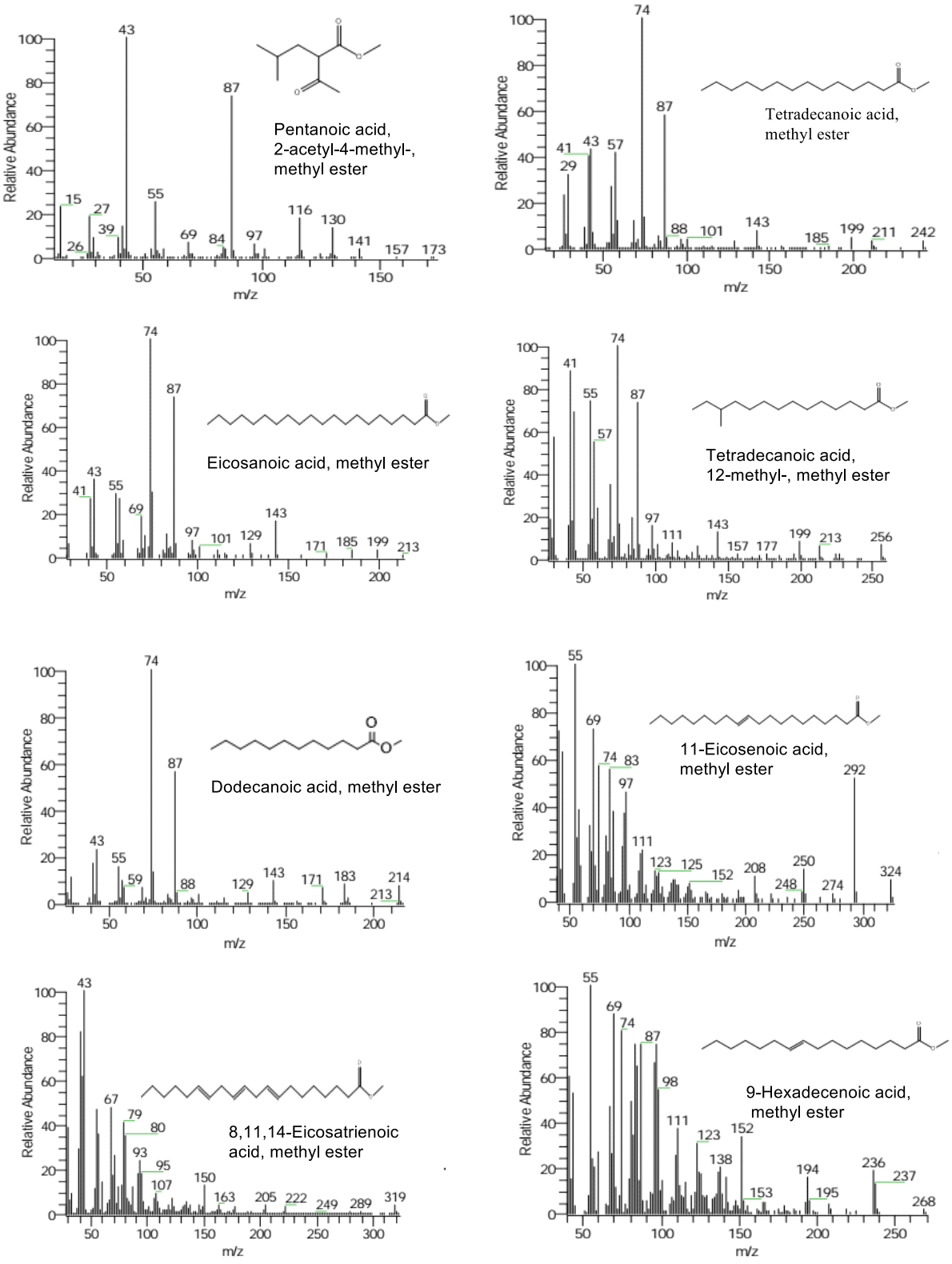


Figure 3: Mass spectra of some phytochemicals listed in table 1.

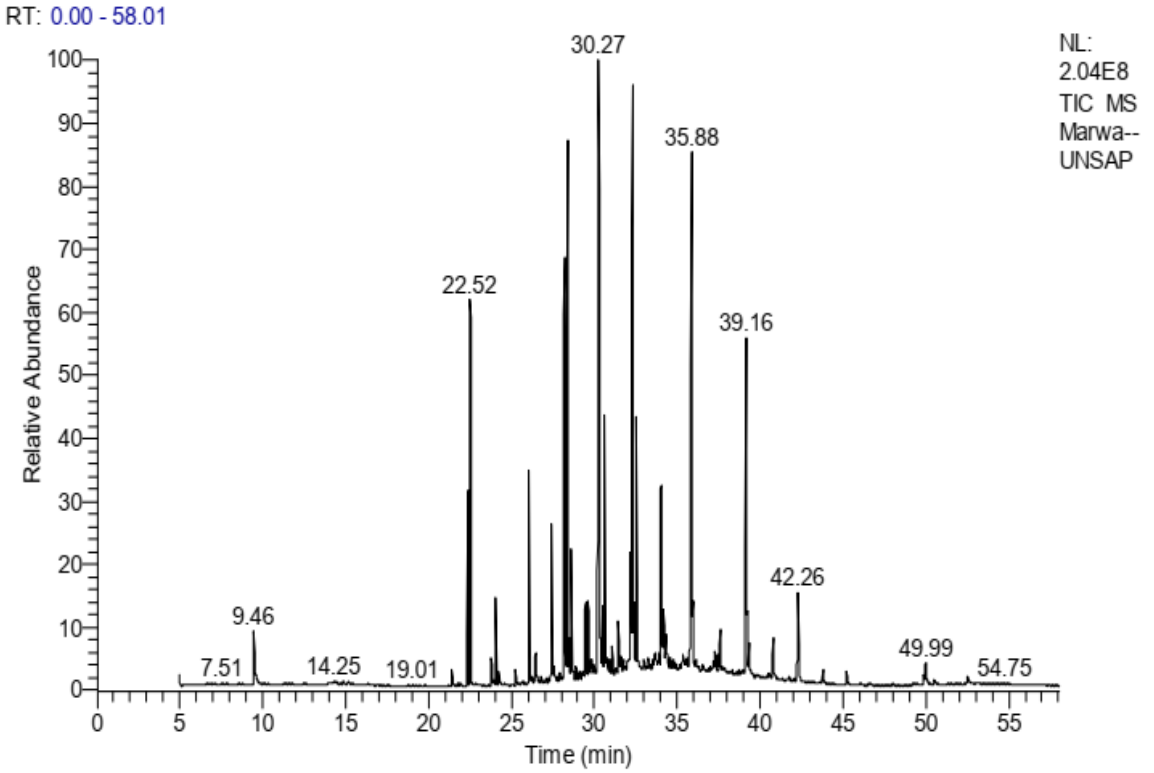


Figure 4: Chromatogram of GC-MS analysis of unsaponifiable matter of sea grass *Thalassodendron ciliatum*.

11-octadecenoic acid, 23-methyl-tetracosa-5,9-dienoic acid, 13-methyl-tetradecanoic acid, nonadecanoic acid, 9,12-hexadecadienoic acid and heneicosanoic acid were also detected. In addition, methyl esters of dodecanoic acid (lauric acid), 14-methyl-pentadecanoic acid, 2-hydroxy-hexa-decanoic acid, 8,11,14-eicosatrienoic acid, 11-eicosenoic acid, 22-tricosenoic acid and 15-tetracosenoic acid (nervonic acid) were also present in traces. Figure 3 represents the mass spectra of selected phytochemicals that listed in Table1.

Tetradecanoic acid, with area percentage of 11.77, possessed antifungal, antibacterial and hypercholesterolemic activities (Elaiyaraja and Chandramohan, 2016). Moreover, it showed a larvicidal activity against *Aedes aegypti* and this was highly supported by other study of a larvicidal activity of 12-methyl- tetradecanoic acid derivative, with % area of 6.07, against Polychaete *Hydroides elegans* (Xu et al., 2009; Sivakumar et al., 2011). Among series of tested saturated fatty acids, lauric acid and myristic acid were the most active bactericides against Gram negative bacteria but with lower activity than unsaturated fatty acids (Galbraith et al., 1971). In general, it is known that long-chain unsaturated fatty acids inhibit bacterial growth by disruption of bacterial fatty acid synthesis. Unsaturated fatty acids are more

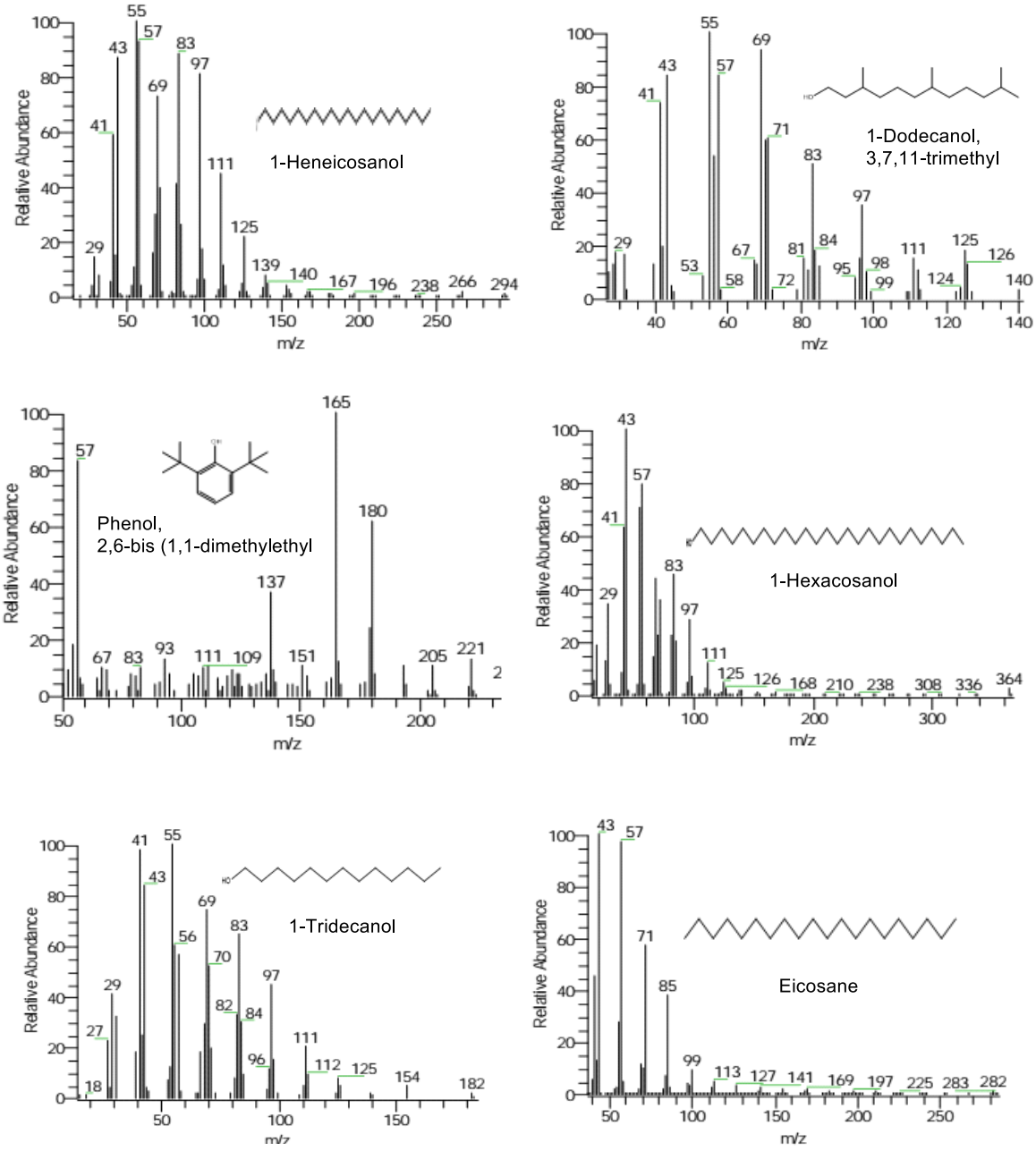


Figure 5: Mass spectra of some phytochemicals listed in table 2.

active than the corresponding saturated fatty acids. The bacterial growth inhibition is enhanced with the increase of double bonds present in the molecules (Skalicka-Woz´niak et al., 2010). Based on this, our reported unsaturated long chain fatty acids, 5,9-docosadienoic acid, 23-methyl-tetracosa-5,9-dienoic acid, 9,12-Hexadecadienoic acid and 8,11,14-eicosatrienoic acid, may have antibacterial inhibition activity and further biological studies are needed. Moreover, unsaturated fatty acids are essential for normal growth of cells especially of blood vessels and nerves, integrity of cell structure, as well as the ability to lower cholesterol levels of the blood (Igwe and Okwu, 2013).

Both saturated fatty acids of chain lengths C-8 to C-18 and unsaturated fatty acids of chain length C-11 to C-24 were assessed for their antioxidant capacity. The saturated fatty acids, octanoic (C-8) to undecanoic acid (C-11) did not exhibit antioxidant activity. However, lauric acid, tridecanoic acid and myristic acid, displayed 60, 85, and 71% antioxidant activities, respectively. For unsaturated long chain fatty acids of C-20, cis-11-eicosenoic acid was the most active antioxidant fatty acid followed by 8,11,14-eicosatrienoic acid which manifested also a significant inhibition of COX-II enzyme activity, inflammation causing enzyme (Henry et al., 2002). Furthermore, palmitoleic acid (9-hexadecenoic acid) present in a considerable amount in *T. cilliatum* saponified part (4.86 %) as shown in table 1, was reported previously to exert strong anti-inflammatory effect (Astudillo et al., 2017)

GC-MS analysis of unsaponifiable matter

The GC chromatogram of the unsaponified fraction of *T. ciliatum* hexane extract (Figure4) demonstrated the presence of 59 compounds. The identified constituents consisted of 68% oxygenated compounds and 32% unoxygenated ones. Some of the determined compounds were listed in table 2 based on their predominance or previously reported biological activity. From table 2, we noticed that 1-heneicosanol was the most predominant volatile phytochemical with % area of 13.94. 1-Heneicosanol showed a significant antifungal and antibacterial activities against *Candida albicans* and *C. krusei, Staphylococcus aureus* and *Pseudomonas aeruginosa* (Arancibia et al., 2016). Likewise, 3,7,11-trimethy-l1-dodecanol, known as hexahydrofarnesol, was also major phytochemical with % area of 12.44 followed by 1,1'-oxybis-octane with % area of 10.16. Other peaks were determined like penta-3,4-dienoic acid, 1-pentadecanol, 2,6- bis (1,1-dimethylethyl) phenol, 1-hexacosanol, 2-heptadecanone, 1-octadecanol, 1-tridecanol, cyclotetradecane, 2,3-dicyano-7,7-dimethyl-5,6-benzonorbornadiene, 1-hexadecanol, 2-hexadecanone, 6,10,14-trimethyl-2-pentadecanone, eicosane and 1-methyl-1-(1-methylethyl)-2-nonyl-cyclopropane. Few steroidal compounds were identified as cholestane-3-ol and stigmast-5-en-3-ol. According to previous studies, 2,6-bis (1,1-dimethylethyl) phenol exhibited anti-inflammatory, antifungal, antimicrobial, antioxidant, antimalarial activities (Costantino et al., 1993; Elaiyaraja and Chandramohan, 2016). 1-Hexacosanol was found to have larvicidal activity against *Chromolaena odorata* through acetylcholinesterase inhibition mechanism (Gade et al., 2017). 1-Dodecanol and 1-tridecanol could be

useful for the natural mosquito control agents against *Aedes aegypti* (Tabanca et al., 2014). Belakhdar and coworkers proved the antibacterial, antimicrobial and cytotoxic activity of eicosane (Belakhdar et al., 2015).

Mass spectra of some selected phytochemicals were shown in figure 5.

4. Conclusion

To the best of our knowledge this is the first reported investigation of *T. ciliatum* lipoidal content. Our results of GC/MS analysis revealed the presence of saturated fatty acids, tetradecanoic acid (myristic acid) and dodecanoic acid (lauric acid), in addition to unsaturated fatty acids, 8,11,14-eicosatrienoic acid, 11-eicosenoic acid and 9-hexadecenoic acid (palmitoleic acid) in the saponifiable part of *T. ciliatum* hexane fraction. Moreover, the following volatile phytochemical compounds were detected; 1-heneicosanol, 2,6-bis (1,1-dimethylethyl) phenol, eicosane, 1-hexacosanol and 1-tridecanol.

These phytochemicals may be the responsible for the reported antimicrobial, antioxidant and anti-inflammatory activities of sea grass *T. ciliatum*

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Conflict of interest

There is no conflict of interest

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