Journal of Sustainability Science and Management
Volume 12 *Number* 2, December 2017: 8-16
ISSN: 1823-8556

FATTY ACIDS COMPOSITIONS OF *Sargassum granuliferum* AND *Dictyota dichotoma* AND THEIR ANTI-FOULING ACTIVITIES

KAMARIAH BAKAR[1], HABSAH MOHAMAD[1]*, JALIFAH LATIP[2], HOCK SENG TAN[3] AND GAN MING HERNG[4]

[1]Institute of Marine Biotechnology, [3]Institute of Oceanography and Environment, [4]School of Marine & Environmental Sciences, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia. [2]School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.

**Corresponding author: habsah@umt.edu.my*

Abstract: In this study, characterization of fatty acids in *Dictyota dichotoma* and *Sargassum granuliferum*, the sea-weeds collected from Pulau Nuyunan, Sabah, were carried out. Fatty acid methyl-ester analysis (FAME) showed palmitic acid, elaidic acid, stearic acid, *cis*-11,14,17-eicosatrienoic acid and erucic acid were dominant in both seaweeds with co poly- and mono-unsaturated fatty acids were higher than the saturated fatty acids. The pure compounds isolated from *Sargassum granuliferum* were hexadecanoic acid, pentadecanoic acid, docosanoic acid, tetracosanoic acid, octadecanoic acid, eicosanoic acid and oleamide. Meanwhile, hexadecanoic acid, tetradecanoic acid and 2-hydroxyhexadecanoic acid were isolated from *Dictyota dichotoma*. All fatty acids isolated exhibited anti-fouling properties with broader activities shown for hexadecanoic acid and fatty acids isolated from *Sargassum granuliferum* and also more superior than *Dictyota dichotoma.*

Keywords: Fatty acids*, Dictyota dichotoma*, *Sargassum granuliferum*, anti-fouling.

Introduction

Seaweeds are rich sources of beneficial bio-active compounds such as carotenoids, *n*-3 fatty acids, and polyunsaturated fatty acids (PUFA) which are unique features of the lipids from marine organisms (Stengel *et al*., 2011). The benefits and potentials for nutritional and medical applications have made marine algae as the subject of important research in the last two decades (Khomtichenko, 1991; Zhukova & Svetashev, 1999; Wong & Cheung, 2000; Kim *et al*., 2010; Kendel *et al*., 2015). The high content of PUFAs, specifically α-linolenic (18:3*n*-3), octadecatetraenoic (18:4*n*-3), arachidonic (20:4*n*-6), and eicosapentaenoic acids (20:5*n*-3) (Dawczynski *et al*., 2007) in marine algae have drawn great interest as this class of acids is considered essential nutritional components in humans and animals (Rustan & Drevon, 2005). They may have important roles in the prevention of cardiovascular diseases and cancer, exhibiting both anti-athrogenic and anti-thrombotic effects as well as in the control of rheumatoid arthritis and hypertension (Mahaffey, 2003; Sidhu, 2003; Tsape *et al*., 2010). Lipids profiling could assist in the assignment of algal taxonomic position and in providing the signature profiles for use in organic geochemistry and food studies (Rajasulochana *et al*., 2010).

Marine bacteria such as *Vibrio alginolyticus*, *V. mimicus*, *V. parahaemolyticus*, *Pseudomonas aeruginosa* and *Bacillus subtilis* are known to exhibit strong biofilm forming ability (Diaz *et al*., 2015; Abu Sayem *et al*., 2011). Extensive studies on novel anti-fouling compounds against biofouling have been conducted (Holmstroem & Kjelleberg, 1999; Kitano *et al*., 2011). Marine natural products are considered as promising sources of anti-fouling compounds and these include the fatty acids, terpenoids, polyethers and alkaloids (Qian *et al*., 2010). They are also biodegradable and will be of great interest in the development of anti-fouling paint for ships and marine infrastructures. The screening for anti-fouling bioactivity is important for the development of anti-foulant from natural products library originated from marine organisms in Malaysia.

Sargassum and *Dictyota* are classified as brown algae (class Phaeophyceae). The

distribution of *S. granuliferum* in the West Coast of Peninsular Malaysia has already been reported (Phang, 2006; Phang *et al*., 2016). However there is no report on the distribution of *S. granuliferum* from Pulau Nuyunan Laut, Sabah, Malaysia. The *S. granuliferum* is actually closely related to *S. myriocystum* and the fatty acids constituents of both species have not been reported elsewhere. In the case of *D. dichotoma*, the fatty acid constituents for the species distributed around the globe has been reported before (Chu *et al*., 2003, Gosch *et al*., 2012), but none from Pulau Nuyunan Laut.

In this study, characterization of fatty acids in *D. dichotoma* and *S. granuliferum* collected from Pulau Nuyunan Sabah, was carried out. In addition, the anti-fouling potential of pure fatty acids compound isolated from these samples was investigated.

Materials and Methods

Seaweeds Collection and Preparation

Sampling of healthy and matured seaweeds (*D. dichotoma* and *S. granuliferum*) was carried out at Pulau Nunuyan Laut, Sandakan, Sabah (05˚56.030´N; 118˚06.609´E) on 26th March 2008 and 15th June 2008 via scuba diving at the depth of 8-15 m by Mr Gan Ming Herng. Immediately after collection, all seaweed samples were washed with fresh seawater to remove epiphytes, sand and other extraneous matter. Further, the samples were washed thoroughly using tap water to remove the surface salt. The water was drained off and the samples were air dried under shade for a period of one week. Once dried, all seaweeds samples were kept in plastic bags and sealed tightly until further analysis. The species were identified (voucher specimens MARC08P0054-P0055, MARC08P0056-P0056$_{iv}$, MARC08P0073), and their herbariums were deposited at Institute of Oceanography and Environmental (INOS), Universiti Malaysia Terengganu.

Extraction and Isolation of S. granuliferum and D. dichotoma

Dried sample of *D. dichotoma* and *S. granuliferum* were cut into small pieces and ground into powder form using metal grinder. Dried powder of *D. dichotoma* (2 kg) and *S. granuliferum* (2.3 kg) were soaked separately with 2.5 L of methanol and shaken using orbital shaker at the speed of 120 rpm for a period of three days. Soaked samples were then filtered to remove solid particles from the solvent extracts. These procedures were repeated thrice and the sample extracts were evaporated using a rotary evaporator. The extrated samples were then re-extracted with petroleum ether, chloroform, ethyl acetate and butanol, with similar process as mentioned above.

The isolation and purification of pure compounds from methanol, petroleum ether, chloroform, ethyl acetate and butanol extracts of *S. granuliferum* and *D. dichotoma* were done using column chromatography and preparative thin layer chromatography (TLC) techniques. Table 1 shows the different solvent extracts for *S. granuliferum* and *D. Dichotoma*.

Table 1: Different solvents for *S. granuliferum* and *D. dichotoma* extracts classificatio

Type of Solvent	Seaweed Samples	
	S. granuliferum	***D. dichotoma***
Methanol	SGM	DDM
Petroleum Ether	SGP	DDP
Chloroform	SGC	DDC
Ethyl Acetate	SGE	DDE
Buthanol	SGB	DDB*

Note: - : DDB* buthanol extract was obtained for *D. dichotoma* in low amount, not eligible for further test

Methylation of Fatty Acids

Sample was first added with 9 mL of $CHCl_3$/MeOH (1:2) in a centrifuge tube, vortexed for 30 s, followed by the addition of 12 mL of $CHCl_3$. The solution was then vortexed for 30 s, and later added with 24 mL of distilled water, and vortexed. Aqueous layer was discarded, whilst organic layer was transferred into a 150 mL round bottom flask. The procedures were repeated thrice until a clear organic layer was obtained. The collected organic layer was dried using rotary-evaporator under controlled temperature and pressure. Two mL of MeOH or NaOH solution (0.5 M) was added to the sample and saponified for 2 hours. Then, 2 mL of 10% $KHSO_4$ or $NaHSO_4$ and 2 mL of phenanthrene solution was added. The sample mixture was shaken rigourously and let to rest until two visible layers were observed. The bottom layer was discarded while the top layer was recovered using Pasteur pipette and kept in a vial.

Fatty Acids Methyl Ester (FAME) Analysis

Identification and quantification of the fatty acid methyl esters (FAME) and sterols in *D. dichotoma* and *S. granuliferum* were carried out using Shimadzu-QP2010 GC-MS. FAME compounds determined included the saturated and unsaturated (mono and poly) fatty acids ranging from butyric methyl esters (C4:0) to lignoceric acid methyl esters (C24:0). The sum of saturated fatty acids is referred to as total saturated fatty acid (TSFA), the mono-unsaturated fatty acids as total mono-unsaturated fatty acid (TMUFA), and the poly-unsaturated fatty acids as the total poly-unsaturated fatty acid (TPUFA). The GC-MS operating conditions for FAME were summarized in Table 2. Verification of peaks was based on key fragment ions, retention times in comparison to the external standards of fatty acids methyl esters (Supelco™ 37 FAME mix) and/or mass spectra from published literature.

Anti-fouling Assay

The crystal violet biofilm assay method was based on the micro titer plate test as previously described (Stepnovic *et al*., 2000), with some modifications. In this assay, 5 bacterial strains (*Vibrio alginolyticus, Vibrio mimicus, Vibrio parahaemolyticus, Pseudomonas aeruginosa, Bacillus subtilis*) were chosen for their ability to form biofilm. Steriled 96-well polypropylene tissue culture plates with flat bottom were

Table 2: Description of GCMS conditions

No.	Parameter	Description
1	Temperature of oven	50 °C (held for 1 min), then up to 140 ºC at 8 ºC/min, then up to 300 °C at 7 °C/min and held isotherm for 10 min
2	Mode	Full-scan GCMS
3	Column	DB-5 (5% diphenyldimethylpolysiloxane; 30m long, 0.25 mm I.D., 0.25 µm film thickness)
4	Carrier gas	Helium (purity 99.999%)
5	Column flow	1.20 mL/min
6	Nominal initial pressure	18.8 kPa
7	Linear velocity	44.4 cm/sec
8	Injection mode	Splitless (1 min) (1.0-1.4 µL; hot needle technique)
9	Injector temperature	290 °C
10	Transfer line temperature	300 °C
11	Data for qualitative analysis	Acquired in the electron impact (EI) mode (70eV), scanning from 50-650 atomic mass units

prepared by filling with 230 µL of Luria-Bertani (LB) broth. Twenty µL of bacteria was added separately into different wells. Triplicate test was carried out for each bacteria. Different compounds isolated from *S. granuliferum* and *D. dichotoma* were added at the specified concentrations. Negative control well was also included for bacterial culture without compounds addition.

The plates were incubated aerobically on a horizontal shaker (120 rpm), at 37 °C for 24 h for biofilm development. The content of the plate was then washed twice with 200 µL/well of sterilized water to remove weakly adhered cells. The attached mass of biofilm was quantified using crystal violet staining (Gram colour staining set for microscopy; Merck). Briefly, the plates containing the biofilms were set to air dry for 30 min, and 200 µL of absolute methanol was transferred to each well, in order to fix the adhered cells and allowed to contact for 15 min. Then, the methanol was removed and 200 µL of crystal violet (1% v/v) per well was added. The plate was then washed with sterilized distilled water and placed at room temperature for one hour. After the plates were air dried, the dye bound to adherent cells was re-solubilized with 200 µL of 33% (v/v) glacial acetic acid added to each well and agitated in ELISA reader for 15 min. The crystal violet solutions obtained were transferred to a new sterile 96-well plate and the optical density (OD) was measured using ELISA reader at 595 nm.

Results and Discussion

Fatty Acid Methyl Ester in S. graniferum and D. dichotoma

The profile of individual fatty acid detected in different extracts of *D. dichotoma* and *S. granuliferum* is given in Table 3. Prior to FAME analysis, all the solvent extracts as listed in Table 1 was first converted to fatty acid methyl ester (FAME) as the non-volatile properties of fatty acids require methylation prior to gas chromatography analysis (Christie, 1998; Kawashima *et al*., 2008; Prabhakar *et al*., 2011). In *S. granuliferum* extracts, the most dominant fatty acid was palmitic acid as exhibited in SGM (39.5%), SGB (35.2%), SGC (32.8%), and SGE (31.6%), whilst, elaidic acid (26.2%) and palmitic acid (23.8%) in SGP extracts were dominant. All types of *S. granuliferum* extracts showed the presence of myristic, *cis*-10-pentadecenoic, palmitic, palmitoliec, oleic, linoleic and lignoceric acids. The percentage of unsaturated fatty acids was higher than saturated fatty acids in SGC, SGP, SGE and SGB, whereas it is vice versa in SGM. The TMUFA were also higher than the TSFA and TPUFA in SGC, SGP and SGE. For SGM and SGB, the TSFA was the highest.

Palmitic acid was also dominant in DDE (43.6%) and DDC (24.9%) extracts. Major fatty acids in DDM were *cis*-5,8,11,14,17-eicosapenteanoic acid (19.7%) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (18.2%) whilst erucic acid (49.1%) was a major constituent in DDP. High percentage PUFA and MUFA was shown in DDC (62.9%), DDM (88.0%) and DDP (90.7%), whilst DDE was dominated by SFA (68.0%). Both DDM and DDC displayed higher PUFA (69.6% and 51.3%, respectively), than MUFA (18.4% and 11.6%, respectively) and SFA (11.9 and 37.1%, respectively). The fatty acid profile in DDP was in the order of MUFA>PUFA>SFA, whilst DDE was in the order of SFA (68%) > MUFA (25.7%) > PUFA (6.3%). *Cis*-5,8,11,14,17-eicosapenteanoic acid (EPA) (19.7%), *cis*-8,11,14-eicosatrienoic acid (14.7%) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) (18.2%) were among the major PUFAs in DDM.

Overall results suggested that palmitic acid was the major fatty acid in both *S. granuliferum* and *D. dichotoma*. Palmitic acid is infact the dominant fatty acids found in the seven *Sargassum* species which include *S. miyabei, S. pallidum, S. herklotsii, S. baccularia, S. microcsytum, S. turbinariodes, S. cristaefolum* (Khotimchenko, 1991), three *Dictyota* species (*D. dichotoma, D. hauckiana, D. indica*) (Shaikh *et al*., 1991), and *Ulva lactuca, Sargassum wightii,* and *Kappaphycus alvarezii* (Muralidhar *et al*., 2010). Similar findings have been reported for three algae classes- phaeophyte (*Egregia*

menziessii), rhodophyte (*Chondracanthus canaliculatus*) and chlorophyte (*Ulva lobata*) (Nelson *et al*., 2002). Palmitic acid is also found not changing with different seasons (Nelson *et al*., 2002), thus confirming the dominance of palmitic acid in seaweeds.

Erucic acid, a potential component for bio-diesel was detected in high percentage in DDP. In *S. granuliferum* extract, the percentage of erucic acid was generally low and was not detected at all in SGP. Both *S. granuliferum* and *D. dichotoma* extracts also showed higher total unsaturated fatty acid contents than the saturated ones, except for DDE and SGM. Also, *S. granuliferum* showed higher percentage of fatty acids than *D. dichotoma*. Earlier studies show predominance of unsaturated fatty acids in algae (Prabhakar *et al*., 2011; Nelson *et al*., 2002; Easa *et al*., 1995) but the percentage of saturated over unsaturated fatty acids depend on the seaweeds species and the location of the samples taken (Gosch *et al*., 2012).

Table 3: Profile of fatty acids in different extracts of *S. granuliferum* and *D. dichotoma*

Fatty acid	FAME in different extracts (%)								
	S. granuliferum					*D. dichotoma*			
	SGM	**SGP**	**SGC**	**SGE**	**SGB**	**DDM**	**DDP**	**DDC**	**DDE**
Myristoleic acid (C14:1)	1.6	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.9
Myristic acid (C14:0)	6.2	3.9	4.1	4.3	4.5	1.2	1.6	n.d	10.1
cis-10-Pentadeccanoic acid (C15:1)	4.9	1.7	4.7	1.6	1.5	0.6	0.8	n.d	1.1
Pentadecanoic acid (C15:0)	0.5	n.d	0.5	0.4	0.3	n.d	n.d	n.d	2.1
Palmitoleic acid (C16:1)	8.3	9.9	8.5	8.0	17.3	1.9	1.7	n.d	n.d
Palmitic acid (C16:0)	39.5	23.8	32.8	31.6	35.2	3.8	6.7	24.9	43.6
cis-10-Heptadecanoic acid (C17:1)	n.d	n.d	n.d	2.7	1.9	1.1	n.d	n.d	n.d
Heptadecanoic acid (C17:0)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
γ-Linolenic acid (C18:3n6)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.6
Linolelaidic acid (C18:2n6t)	n.d	n.d	n.d	n.d	6.8	1.0	1.6	n.d	1.2
Linolenic acid (C18:3n3)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	21.6	n.d
Linoleic acid (C18:2n6c)	3.4	3.9	3.5	2.8	21.5	3.2	6.1	n.d	n.d
Elaidic acid (C18:1n9t)	n.d	26.2	21.3	20.5	n.d	n.d	n.d	n.d	10.2
Oleic acid (C18:1n9c)	20.8	3.6	3.0	2.7	4.8	n.d	n.d	n.d	9.5
Stearic acid (C18:0)	1.3	n.d	1.1	n.d	1.0	0.6	1.1	12.1	7.2
Arachinodic acid (C20:4n6)	n.d	n.d	1.6	1.1	3.1	3.7	n.d	10.1	n.d
cis-5,8,11,14,17-Eicosapenteanoic acid (C20:5n3)	1.5	2.8	n.d	4.4	0.8	19.7	3.9	n.d	2.1
cis-8,11,14-Eicosatrienoic acid (C20:3n6)	n.d	n.d	n.d	n.d	n.d	14.7	n.d	n.d	n.d
cis-11,14-Eicosadienoic acid (C20:2)	n.d	1.1	n.d	n.d	n.d	n.d	n.d	3.8	n.d
cis-11-Eicosenoic acid (C20:1)	2.8	3.3	7.2	6.3	n.d	n.d	n.d	n.d	2.6
cis-11,14,17-Eicosatrienoic acid (C20:3n3)	n.d	2.6	5.3	n.d	n.d	9.1	15.8	6.2	2.4
Arachidic acid (C20:0)	0.5	0.4	1.3	0.6	0.2	6.3	n.d	n.d	n.d
Heniecosanoic acid (C21:0)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
cis-4,7,10,13,16,19-Docosahexaenoic acid (C22:6n3)	n.d	15.3	n.d	8.3	n.d	18.2	n.d	9.7	n.d
cis-13,16-Docosadienoic acid (C22:2)	n.d	n.d	n.d	n.d	n.d	n.d	11.6	n.d	n.d
Erucic acid (C22:1n9)	4.0	n.d	2.9	4.2	0.5	14.8	49.1	5.7	1.3
Behenic acid (C22:0)	0.6	0.9	1.0	n.d	0.4	n.d	n.d	n.d	n.d
Tricosanoic acid (C23:0)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Nervonic acid (C24:1)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	5.9	n.d
Lignoceric acid (C24:0)	4.2	0.5	1.1	0.4	0.3	n.d	n.d	n.d	n.d
Total Saturated Fatty Acid (TSFA)	52.7	29.5	41.9	37.2	41.9	11.9	9.3	37.1	68.0
Total Mono-Unsaturated Fatty Acid (TMUFA)	42.4	44.8	47.7	46.1	26.0	18.4	51.7	11.6	25.7
Total Poly-Unsaturated Fatty Acid (TPUFA)	4.8	25.7	10.5	16.7	32.1	69.6	39.0	51.3	6.3

Note: The abbreviations are as explained in Table 1
n.d= not detected

Anti-fouling Activity of Purified Fatty Acids

The purified hexadecanoic acid, pentadecanoic acid, docosanoic acid, tetracosanoic acid, octadecanoic acid, eicosanoic acid, tetradecanoic acid and 2-hydroxyhexadecanoic acid were subjected to anti-fouling crystal violet biofilm assay (Table 4) against five marine bacteria - *V. Alginolyticus*, *V. Mimicus*, *V. Parahaemolyticus*, P. *Aeruginosa* and *B. Subtilis*. All bacteria were selected due to their strong biofilm forming ability as described previously (Abu Sayem *et al*., 2011; Diaz *et al*., 2015). Hexadecanoic acid showed activities in all the five bacteria tested with the highest inhibition activity against *V. alginolyticus* and *B. subtilis* at IC_{50} values of 60.57 and 126.66 ± 0.13 µg/mL, respectively. The next highest was Tetradecanoic acid against *V. parahaemolyticus* and *P. aeruginosa* at IC_{50} values of 69.2 and 122.9 µg/mL, respectively. 2-hydroxyhexadecanoic acid showed activity only against *V. alginolyticus* but no activity with all other bacteria tested. Docosanoic acid, octadecanoic acid and tetradecanoic acid showed activities against three types of bacteria - *V. alginolyticus* (28.545 ± 0.06; 501.23 ± 0.11; 145.16 ± 0.07)*, V. parahaemolyticus* (481.60 ± 0.05; 275.99 ± 0.04; 69.20 ± 0.14) *and P. aeruginosa (140.41* ± 0.04; 587.80 ± 0.10; 122.94 ± 0.08), respectively.

Palmitic acid is reported to possess anti-bacterial activity and could inhibit the growth of biofoulers diatom *Cylindrotheca closterium* and the germination of *Ulva lactuca* spores. Interestingly this compound is not toxic to untargeted marine organism involved in the biofouling process (Bazes *et al*., 2009). Thus palmitic acid is a good lead towards the development of an environmentally friendly anti-fouling paint. Several stuctural modification has been done to incoorporate palmitic acid moeity in the anti-biofilm chemicals. This has led to the synthesis of potential anti-fouling agents such as N-(4-hydroxy-3-methoxybenzyl)-hexadecanamide (Iswatun, 2015). A compound containing palmitic acid residue such as 1-*O*-palmitoyl-2-*O*-oleoyl-3-*O*-(α-D-glucopyranosyl)-glycerol also posseses anti-fouling property (Plauguerne *et al*., 2014).

Table 4: Anti-fouling activity of pure fatty acids isolated from *S. granuliferum* and *D. dichotoma*

Sample	Pure Compounds	IC_{50} (µg/mL)				
		Bacteria				
		VA	***VM***	***VP***	***PA***	***BS***
S. granuliferum	Hexadecanoic acid / Palmitic acid	60.57±0.09	220.63±0.03	132.03±0.09	309.55±0.06	126.66±0.13
	Pentadecanoic acid	240.45±0.08	NA	182.93±0.07	NA	NA
	Docosanoic acid / Behenic acid	285.54±0.06	NA	481.60±0.05	140.41±0.04	NA
	Tetracosanoic acid / lignoceric acid	210.46±0.92	NA	312.50±0.07	NA	NA
	Octadecanoic acid / stearic acid	501.23±0.11	NA	275.99±0.04	587.80±0.10	NA
	Eicosanoic acid / arachidic acid	265.29±0.18	NA	NA	116.59±0.00	NA
D. dichotoma	Hexadecanoic acid / palmitic acid	233.25±0.52	NA	318.41±0.02	116.50±0.09	NA
	Tetradecanoic acid / myristic acid	145.16±0.07	NA	69.20±0.14	122.94±0.08	NA
	2-hydroxyhexadecanoic acid	296.01±0.11	NA	NA	NA	NA

Abbreviation: NA - Not Active, *VA=Vibrio alginolyticus*, *VM= Vibrio mimicus*, *VP= Vibrio parahaemolyticus*, *PA= Pseudomonas aeruginosa*, *BS= Bacillus subtilis*.

Conclusion

Fatty acids can play important role in the defence and protection against bio-film forming bacteria. *S. granuliferum* and *D. dichotoma* showed high content of poly-unsaturated fatty acid (PUFA) with Hexadecanoic acid as the dominant fatty acid detected in both. Isolation of pure fatty acid compounds showed *S. granuliferum* having more types of fatty acids than *D. dichotoma*. The purified compounds were hexadecanoic acid, pentadecanoic acid, docosanoic acid, tetracosanoic acid, octadecanoic acid, eicosanoic acid, tetradecanoic acid, and 2-hydroxyhexadecanoic acid with simple structure of each compound showing a promising candidate as new antifouling additive. The high content in important fatty acids especially the SFA and MUFA make both species good candidates for biodiesel, and PUFA for nutritive food source.

Acknowledgements

The authors are grateful to the Institute of Marine Biotechnology (IMB) for the facilities. Financial supports from FRGS fund (Vote No: 59161) and the use of GC-MS from the Institute of Oceanography (INOS) is kindly acknowledged.

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