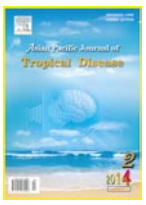
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd



Document heading doi: 10.1016/S2222-1808(14)60769-6

Microalgal fatty acid methyl ester a new source of bioactive compounds with antimicrobial activity

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ARTICLE INFO

Article history:
Received 14 May 2014
Received in revised form 7 Jul 2014
Accepted 13 Jul 2014
Available online 23 Aug 2014

Keywords:
Microalgae
Bioactive compounds
Nostoc spongiforme NTAS04
FAME

ABSTRACT

Objective: To evaluate fatty acid composition and the antimicrobial activity of the major fraction of fatty acid methyl ester (FAME) extracts from three microalgae collected from freshwater lakes in Theni District, Tamil Nadu, India.

Methods: Antimicrobial study was carried out by well diffusion method against bacterial as well as fungal pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Enterobacter* sp., *Klebsiella* sp., *Salmonella typhi*, *Fusarium* sp., *Cryptococcus* sp., *Candida* sp., and *Aspergillus niger* and *Aspergillus flavus*. The FAME profiles were determined through gas chromatography with a flame ionization detector.

Results: The FAME was found to be radial effective in inhibiting the radial growth of both bacterial and fungal pathogens. The FAME extracts exhibited the antibacterial activity against three clinical pathogens, namely, *Escherichia coli*, *Salmonella typhi* and *Enterobacter* sp. with the maximum zone of inhibition of 12.0 mm, 12.0 mm and 11.0 mm, respectively. The FAME showed moderate antifungal activity against *Cryptococcus* sp. (11.8 mm), *Aspergillus niger* (10.5 mm), *Candida* sp. (11.8 mm) and *Fusarium* sp. (10.4 mm). Gas chromatography-flame ionization detector analysis revealed about 30 different FAMEs.

Conclusions: We assume that the observed antimicrobial potency may be due to the abundance of erucic acid methyl ester (C22:0), arachidic acid methyl ester (C20:0), palmitic acid methyl ester (C16:0), cis-11-eicosenoicmethyl ester (C20:1), cis-11, 14-eicosadienoic acid methyl ester (C20:2) and linolenic acid methyl ester (C18:3) in FAMEs which appears to be promising to treat microbial diseases.

1. Introduction

In recent years, many possible sources of natural antibiotics have been in the use for several infectious diseases, mostly bacterial and fungal. Approximately, one-half of deaths in tropical countries are affected severely by infectious diseases[1]. The World Health Organization estimates globally that about 1 500 people die each hour by infectious diseases; half of these are children under five years of age[2].

In the last five decades, increased resistance of bacteria strains to drugs, including antibiotics, has been a major factor increasing morbidity, mortality and health care costs to bacterial infections. It is urgent to discover and develop new infection fighting strategies that counteract the alarming increases in the indiscriminate and abusive use of synthetic antibiotics.

People mostly use synthetic drugs to prevent or control the infectious diseases caused by microbes. Regular use of these drugs leads to development of resistance by the microbes against the drugs[3-5]. It is not only the resistance but also the cost of synthetic chemicals lead to search for alternate medicine such as antimicrobial compounds

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Foundation Project: Supported by the Department of Biotechnology, Government of India (Grant No. BT/PR11316/PBD/26/164/2008).

from natural sources. Plant derived natural products and antibiotics are found to be the effective alternative recognized from natural resources. At this time, essential pharmacological and therapeutic products are being obtained and actively sought from the ocean[6-12]. One of the potential groups of natural resource is algae which are known to possess promising novel bioactive substances[13-15].

Use of microalgae for human consumption as a source of high value health food, biochemical products, such as vitamins, carotenoids, phycocyanin and polyunsaturated fatty acids including the omega-3 fatty acids have been developed. A large number of studies on the microalgal bioactive compounds have oriented to the anti-inflammatory, antiviral, antimicrobial, antihelmintic, cytotoxic, immunological and enzyme inhibition properties.

The applications of microalgal fatty acids are extensively studied. It is surprising that no investigations were made on their antimicrobial activity. However, the antibacterial activities of long chain unsaturated fatty acids have been well known for many years. Fatty acids functioned as the key ingredients of antimicrobial food additives which inhibit the growth of bacterial pathogens. Additionally, long chain unsaturated fatty acids such as linoleic acid and oleic acids are bactericidal to important pathogenic microorganisms, such as methicillin resistant *Staphylococcus aureus* (*S. aureus*)[16], *Helicobactor pylori*[17], and *Candida albicans*[18], but almost no investigations were made on the antimicrobial activity of microalgae fatty acid methyl ester (FAME). In the present study, we report on antimicrobial activities of FAMEs from three species of microalgae from freshwater dam of Theni District.

2. Materials and methods

2.1. Cleaning of glasswares

Clean Borosil glasswares were used. They were soaked in tap water for few minutes and thoroughly washed in tap water. Then they were soaked in dichromate solution for few hours to remove tough residues. They were again washed in tap water.

2.2. Sterilization of glasswares and chemicals

All types of glasswares such as conical flask, Petri plates, test tubes, pipettes, centrifuge tubes, tip boxes, *etc*. and the different types of microalgae cultivated medium such as Chu's 13 medium and BG11 medium were sterilized at 121 °C for 15 min ,15 lbs pressure in autoclave.

2.3. Collection and culturing of microalgae

Microalgae were collected from Manjalar Dam (10°11′-37°15′ N, 77°37′-55°86′ E) of Theni District, Tamilnadu, India during the month of October 2012. The isolation and purification of microalgae were achieved by employing various pure culture techniques. The dominant microalgae were purified and transferred to erlenmeyer flasks containing Chu's 13 medium and BG11 medium[19,20]. They were maintained in germplasm, Department of Microbiology, Bharathidasan University, Tiruchirappalli, India under white fluorescence lamps (3 000 lux), (14±10) L/D at (25±2) °C.

2.4. Preparation of FAME

For each sample, 100 mg of dry lyophilized algae was placed in boiling tubes and added in 50 mL of methanol; chloroform was added (2:1) and mixed vigorously in a shaker for 20 min. The solvent phase was washed with 10 mL water, vortexed and centrifuged at 2 000 r/min for 5 min. The supernatant was removed and the pellet was then rinsed twice with 15 mL methanol and water (1:1). Finally, the extract was collected from the solvent phase, evaporated in a rotary vacuum evaporator and quantified gravimetrically. The weighed lipid and methanolic sulphuric acid (2% H_2SO_4 in methanol) were refluxed for 4 h. The contents were mixed thoroughly with an equal volume of distilled water in separating funnel. Then the aqueous layer was extracted twice with ethyl acetate. The collected ethyl acetate extract containing the FAME was dried over anhydrous sodium sulphate to remove excess moisture and concentrated under vacuum. The dried FAME was analyzed by gas chromatography (GC).

2.5. GC analysis

The FAME samples were analyzed by GC (Shimadzu, GC2014, Japan) with a flame ionization detector (FID). One micro liter of each sample was injected in the FameWax column (Restek, USA) (30 m×32 mm ID×25 mm film thickness). The temperature program was as follows: initial 140 °C with 5 min hold; ramp 2 °C/min to 230 °C with a 5 min hold. Column flow was set at 22.2 mL/min. Instrument conditions were as follows: carrier gas nitrogen; FID set at 260 °C, split ratio of 10:1. Run time for a single sample

was 55 min. Triplicates of each sample were done and FAME identification was run by comparison with standard certificate, Supelco FAME mix C4 –C24 (Bellefonte, PA, USA). Each sample was run under GC in triplicate.

2.6. Antibacterial assay

Antibacterial activity was carried out using the disc diffusion method. The Petri plates were prepared with 20 mL of sterile Mueller Hinton agar (Hi-Media) and the bacterial cultures were swabbed on the top of the solidified media and allowed to dry for 10 min. Four wells were made using a sterile cork borer; samples (FAME, ethyl acetate) were added. Then, the plates were incubated at 37 °C for 24 h.

2.7. Antifungal assay

Antifungal activity was determined by using Sabouraud's dextrose agar. Overnight fungal cultures were used and the well diffusion method was carried out with FAME samples. After inoculation, the plates were incubated at room temperature for 72 h.

3. Results

The FAME of three microalgae extracts, *viz. Nostoc spongiforme* NTAS 04, *Oscillatoria tenius* NTAS 06 and *Chlorococcus* sp. NTAS 12 was determined and the relative percentages are presented in Table 1. In *N. spongiforme* (NTAS 04), the relative percentage of stearic acid (24.44%) was high followed by arachidic acid (7.35%), palmitic acid (2.63%), oleic acid (7.32%), cis-11-eicosenoic acid (12.82%), linoleic acid (19.25%), cis-11, 14-eicosadienoic acid (2.47%) and linolenic acid (8.65%). In *O. tenius* (NTAS 06), the relative percentage of palmitic acid (17.94%) was high followed by heptadecanic acid (5.99%), lignoceric acid (4.13%), behenic acid (2.12%), stearic acid (2.12%), stearic acid (2.12%), cis-10-heptadecanoic acid (10.12%), cis-11-eicosenic acid (17.50%), oleic acid (3.68%), elaidic acid (2.52%), g-linolenic acid (7.39%), cis-11, 14-eicosadienoic acid (8.27%) and cis-13,16-docosadienoic acid (2.05%). In *Chlorococcum* sp. (NTAS 12), the relative percentage of cis-11, 14-eicosadienoic acid (15.70%) was high followed by the arachidic acid (10.67%), linoleic acid (8.55%), linolenic acid (8.82%), erucic acid (7.51%), cis-8, 11, 14-eicosatrienoic (4.58%), cis-10-heptadecanoic (4.57%), pentadecanoic acid (4.37%), behenic acid (4.19%) and olic acid (3.33%). The poly unsaturated fatty acid content of the microalgae was found higher than the saturated fatty acid content (Figure 1).

Table 1
Fatty acid methyl ester composition of microalgae species.

Fatty acid methyl ester		Fatty acid composition (%)		
		NTAS 04	NTAS 06	NTAS 12
Myristic	C14:0	2.12	2.35	2.16
Pentadecanoic	C15:0	1.72	0.00	4.37
Palmitic	C16:0	2.63	17.94	0.00
Heptadecanoic	C17:0	0.40	5.99	1.88
Stearic	C18:0	24.44	2.12	0.00
Arachidic	C20:0	7.35	1.86	10.67
Heneicosanoic	C21:0	1.59	0.00	2.33
Behenic	C22:0	0.00	2.12	4.19
Erucic	C22:0	0.00	2.05	7.51
Tricosanoic	C23:0	0.00	0.00	1.65
Lignoceric	C24:0	0.00	4.13	2.85
Saturated fatty acids		40.25	38.56	37.61
Myristoleic	C14:1	0.37	2.50	0.00
cis-10-Pentadecanoic	C15:1	0.42	0.00	0.00
Palmitoleic	C16:1	1.75	0.49	0.00
cis-10-Heptadecanoic	C17:1	1.73	10.12	4.57
Elaidic	C18:1	0.86	2.52	1.67
Oleic	C18:1	7.32	3.68	3.33
cis-11-Eicosenoic	C20:1	12.82	17.50	0.00
Nervonic	C24:1	0.00	0.00	2.76
Mono unsaturated fatty acids		25.27	36.81	12.33
Linolelaidic	C18:2	3.23	0.00	2.60
Linoleic	C18:2	19.25	0.00	8.55
G-linolenic	C18:3	0.89	7.39	0.00
Linolenic	C18:3	8.65	4.34	8.82
cis-11, 14- Eicosadienoic	C20:2	2.47	8.27	15.70
cis-8, 11, 14-Eicosatrienoic	C20:3	0.00	0.00	4.58
Arachidonic	C20:4	0.00	0.00	2.69
cis-13, 16-Docosadienoic	C22:2	0.00	2.05	3.15
cis-5, 8, 11, 14, 17-Ecosapentenoic	C20:5	0.00	0.70	1.88
cis-4, 7, 10, 13, 16, 19-Docosahexaenoic	C20:6	0.00	1.89	2.09
Poly unsaturated fatty acids		34.49	24.64	50.06
Total		100	100	100

NTAS 04: *N. spongiforme*; NTAS 06: *O. tenius*; NTAS 12: *Chlorococcum* sp.

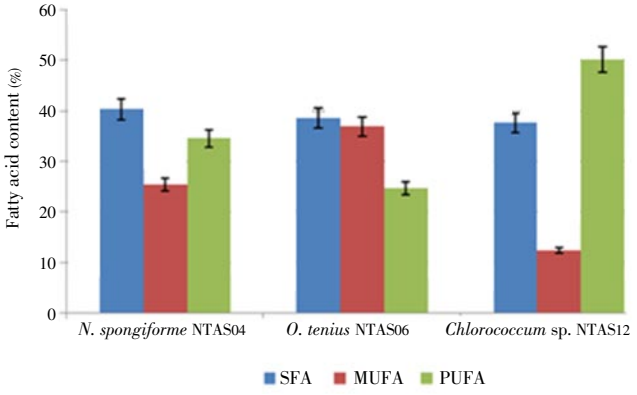

Figure 1. Fatty acid composition of microalgae species.
SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid.

The FAME extract from the three microalgae species possessed significant antimicrobial activity against all the microorganisms tested when compared to the respective positive controls. The FAME extract of *N. spongiforme* (NTAS

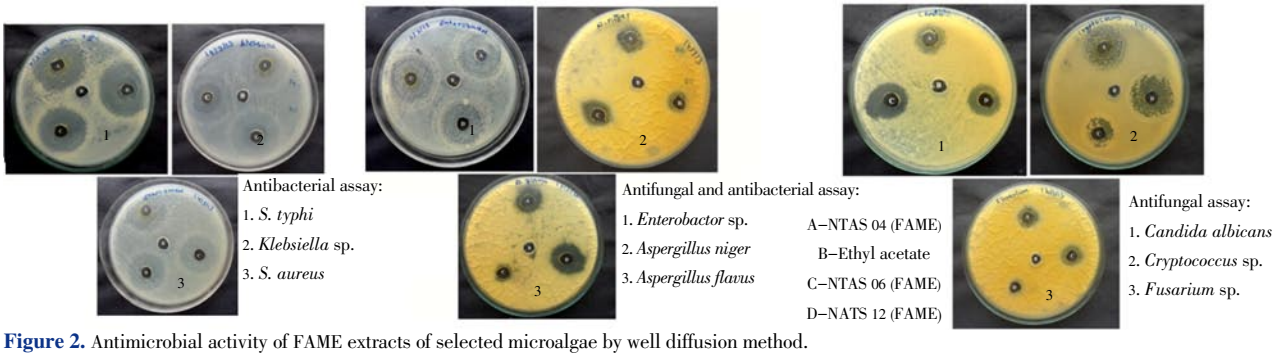

Figure 2. Antimicrobial activity of FAME extracts of selected microalgae by well diffusion method.

04) recorded the highest mean zone of inhibition (12.0 mm) against three clinical pathogens such as *Escherichia coli* (*E. coli*), *Salmonella typhi* (*S. typhi*), *Enterobacter* sp. and minimum zone of inhibition against *Klebsiella* sp. (11.0 mm) was observed. The FAME showed moderate antifungal activity against *Cryptococcus* sp. (11.8 mm), *Candida* sp. (11.2 mm), and *Aspergillus niger* (10.5 mm) (Table 2 and Figure 2).

Table 2
Antimicrobial activity of the FAMEs extracts of microalgae.

Pathogen name	Zone of inhibition (mm)		
	Nostoc sp. NTAS04	*Oscillatoria* sp. NTAS06	*Chlorococcum* sp. NTAS12
S. aureus	11.2	11.4	11.4
Klebsiella sp.	11.0	10.9	10.8
E. coli	12.0	12.0	12.0
S. typhi	12.0	12.0	11.0
Enterobactor sp.	12.0	11.5	11.5
Candida sp.	11.2	11.0	11.8
Cryptococcus sp.	11.8	11.4	11.0
Fusarium sp.	10.7	10.8	10.4
Aspergillus flavus	10.5	11.0	10.5
Aspergillus niger	10.5	11.0	10.8

The FAME extract of *O. tenuis* (NTAS06) also showed the highest zone of antimicrobial activity inhibition against *E. coli* (12.0 mm), *S. typhi* (12.0 mm), and the minimum zone of inhibition against *Klebsiella* sp. (10.9 mm) was observed and showed antifungal activity against *Fusarium* sp. (10.8 mm). The FAME extract of *Chlorococcum* sp. (NTAS12) showed antimicrobial activity the highest zone of inhibition against *E. coli* (12.0 mm), *Enterobacter* sp. (11.5 mm), *S. aureus* (11.4 mm) and minimum zone of inhibition against *Klebsiella* sp. (10.8 mm) was observed and it showed antifungal activity *Candida* sp. (11.8 mm), *Fusarium* sp. (10.4 mm). In contrast, every minimum zone of inhibition was observed in the case of ethyl acetate.

4. Discussion

Antimicrobial active lipids and active fatty acids are present in a high concentration in these microalgae. It was hypothesized that lipids kill microorganisms by leading to the disruption of the cellular membrane as well as bacteria, fungi and yeasts because they can penetrate the extensive meshwork of peptidoglycan in the cell wall without visible changes and reach the bacterial membrane leading to its disintegration[21]. Evidence supporting bioactivity of fatty acids was earlier demonstrated in certain microalgae[22-26]. Microbicidal activity exhibited by microalgae fatty acids may be useful in developing alternative approaches to control different human pathogens, such as those examined in this study.

In spite of extensive studies on the applications of microalgal lipids, the present study discovers the antibacterial and antifungal activity of the FAME. In this study, for the first time the antibacterial and antifungal activity of FAMEs from three microalgal cultures were determined and the extract of *Chlorococcum* sp. showed the highest activity against all the microorganisms tested. The FAME showed activity against both the Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*) along with the fungi *Fusarium* sp. This group of FAMEs was known to inhibit the fatty acid biosynthetic pathway by inhibiting *FabI* gene expression.

These results are in harmony with the work of Lima *et al*. and Canales *et al*. that showed that the methyl esters of fatty acids are endowed with antibacterial and antifungal capacity[27,28]. Hence, the *S. typhi* and *E. coli* were selectively chosen based on their increasing multi drug resistant property, which is a serious clinical problem[29]. Through GC-FID analysis, about 30 different FAMEs were identified, among stearic acid methyl ester (C18:0), erucic acid methyl ester (C22:0), arachidic acid methyl ester (C20:0), palmitic acid methyl ester (C16:0), heptadecanoic acid methyl ester (C17:0), cis-11-eicosenoicmethyl ester (C20:1), cis-11, 14-eicosadienoic acid methyl ester (C20:2) and linolenic acid methy ester (C18:3) gaining high significance due to their strong antimicrobial activity[30]. The antimicrobial activity of erucic acid (C22:0) compared

tocis-11-eicosenoic acid (C20:1) and arachidic acid (C20:0) was found to be higher. The inhibitory activity was in the following order: erucic acid (C22:0)>cis-11-eicosenoic acid (C20:1) and arachidic acid (C20:0). However, palmitic acid methyl ester (C16:0) represents the main constituent of fatty acids and is responsible for the antibacterial activity observed in the target algal species. In several studies, indeed, palmitic acid has been reported to be the major antibacterial compound in a mixture of fatty acids from other algal species. The exact mechanism by which fatty acids exert their bactericidal action remains unresolved but it has been suggested that these molecules initiate per oxidative processes and inhibit bacterial fatty acid synthesis[31]. However, fatty acids may interact with cellular membranes causing leakage of molecules from the cells, reduction of nutrient uptake or inhibition of cellular respiration. Desbois *et al*. and Kabara *et al*. reported that fatty acids such as asoleic, palmitic, stearic, myristic, linoleic and linolenic acids showed activity against *Staphylococcus* sp[32,33]. Nehdi *et al*. and Choi *et al*. have emitted the hypothesis that the antifungal effect was probably due to the predominant lylinoleic acid[34,35]. Greenway and Dyke had suggested that linoleic acid probably inhibited growth by increasing the permeability of the bacterial membrane as a result of its surfactant action[36]. For instance, linoleic and oleic acids were reported as potent antibacterials. Linoleic acid was also stated as a model compound of unsaturated fatty acids, which selectively inhibited FabI enzyme in *S. aureus* and *E. coli*[37].

The results of the present work indicate that the microalgae FAMEs assayed possess antimicrobial properties which could be used on various diseases, through which, the possibility to bring up the most functional and active FAMEs from microalgae shows potential. This report is quick to demonstrate the technical possibility of treating the multidrug resistant pathogens with a new source of antimicrobial products. The valuable outputs of the present study may have a strong imminent into the field of drug discovery.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors are thankful to Bharathidasan University, Tiruchirappalli, India and financial support from the Department of Biotechnology, Government of India is gratefully acknowledged (Grant No. BT/PR11316/PBD/26/164/2008).

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