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## A green synthesis of antimicrobial compounds from marine microalgae *Nannochloropsis oculata*

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## PEER REVIEW

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## Comments

This research work presents an interesting set of data. The obtained results indicate that FAME have a great potential if used as inhibitor of bacteria growth.

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## ABSTRACT

**Objective:** To determine the antibacterial and anti-candidal activities of fatty acid methyl esters (FAME) extracted from marine microalga *Nannochloropsis oculata* and evaluate the inhibition activity of DNA isolated from test pathogenic microorganism.

**Methods:** FAME was synthesized by transesterification of oil using immobilized lipase and characterized using gas chromatography–mass spectrometer. The FAME profile was determined using gas chromatography. The antimicrobial effect was tested by disc diffusion method against Gram–positive bacteria *Staphylococcus aureus*, *Bacillus subtilis*, Gram–negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa* and yeast *Candida albicans*, at varying concentrations of 10, 20 and 30 µL/disc.

**Results:** The results shown that palmitic acid (C16:0), oleic acid (C18:1) and arachidic acid (C20:0) were dominant in *Nannochloropsis oculata* oil. The study revealed that FAME was more active against Gram–negative than against Gram–positive and yeast. DNA inhibition activity results also confirmed that FAME had the bactericidal effect that was revealed by sheared fragments of DNA.

**Conclusions:** The results indicated that microalgal FAME could be potentially utilized as a newer and good source of therapeutic agent in pharmaceutical industry.

## KEYWORDS

*Nannochloropsis oculata*, FAME, Antimicrobial effect, Zone of inhibition, Gas chromatography–mass spectrometer

### 1. Introduction

There are number of clinically efficacious antibiotics becoming less effective due to the development of antibiotic resistant microorganisms[1,2]. It becomes a greater problem to treat many diseases caused by resistant pathogenic microorganisms worldwide. In addition, decreased activity of commonly used antibiotic and resistance of pathogens to such antibiotics have anticipated the development of new alternatives[3]. Marine planktons especially algae are rich source of many interesting bioactive molecules

including lipid which may be useful for the development of antimicrobial drugs[4,5]. Marine microalgae have been an unique source of chemical compounds of pharmaceuticals, aquaculture, cosmetics, anticancer agents, enzymes, pigments, antioxidants, polyunsaturated fatty acids, dietary supplements, agrochemicals and biofuel[6–12].

There are many reports related to antimicrobial activity of crude extracts of marine macro and microalgae[13,14]. From the literature survey we learnt that only a few studies have been reported on inhibitory activity of fatty acid methyl esters (FAME) of marine microalgae and there is no report

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on molecular studies. To the best of our knowledge this is the first report on antimicrobial activity of FAME from marine microalgae produced by an ecofriendly green process using immobilised enzyme system.

The aims of this study were to determine the antibacterial and anti-candidal activities of FAME extracted from marine microalga *Nannochloropsis oculata* (*N. oculata*) and evaluate the inhibition activity of DNA isolated from test pathogenic microorganism.

## 2. Materials and methods

### 2.1. Microalgal culture

*N. oculata*, obtained from Central Marine and Fisheries Research Institute, Tuticorin, Tamilnadu (India), was grown in sterile Walne's medium. The filtered sterilized sea water was enriched with required quantity of Walne's medium containing: NaNO<sub>3</sub>, 100 g/L; NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 20 g/L; Na<sub>2</sub>EDTA, 4 g/L; H<sub>3</sub>BO<sub>3</sub>, 33.6 g/L; MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.36 g/L; FeCl<sub>3</sub>·6H<sub>2</sub>O, 13 g/L; vitamin B<sub>12</sub>, 0.001 g/L and vitamin B<sub>1</sub>, 0.02 g/L. The trace metal solution contained: ZnSO<sub>4</sub>·7H<sub>2</sub>O, 4.4 g/L; CoCl<sub>2</sub>·6H<sub>2</sub>O, 2 g/L; (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·H<sub>2</sub>O, 0.9 g/L; and CuSO<sub>4</sub>·5H<sub>2</sub>O, 2 g/L. The medium was adjusted to pH 8 and autoclaved at 121 °C for 20 min. The filter sterilized vitamins were added after cooling. The contents were later introduced into a 250-mL Erlenmeyer flask and finally transferred to 25 L photobioreactor. Mixing was done by sparging air from the bottom of the photobioreactor; lighting was supplied by four cool-white fluorescent tubes with an intensity of 5000 lux.

### 2.2. Microscopic study of intracellular lipid

The intracellular lipid present in the microalgae was identified by Nile red staining method. A stock solution of Nile red stain (9-diethylamino-5H-benzo (α) phenoxa-phenoxazine-5-one) was prepared according to Mohamady et al<sup>[15]</sup>. About 2.5 mg of Nile red was dissolved in brown bottle containing 100 mL of acetone and this was stored in dark condition. Each 0.5 mL of microalgae culture broth were centrifuged at 1500 r/min for 10 min and the pellets were washed with sterile distilled water (equal volume) for several times. The cell pellets were then mixed with 0.5 mL of Nile red solution incubated for 10 min at room temperature. After washing with distilled water, the stained cells were observed under fluorescence microscopy<sup>[16]</sup>.

### 2.3. Isolation of microalgal oil

*N. oculata* oil was extracted using the method of Bligh and Dyer with slight modifications<sup>[17]</sup>. The biomass suspension was mixed with chloroform: methanol (1:2), vortexed for few minutes and incubated on ice for 10 min. Then, chloroform was added, followed by addition of 1 mol/L HCl and vortexed again for few minutes. Finally, the whole suspension was centrifuged at maximum speed for 2 min. Bottom layer containing lipid was transferred into a fresh previously weighed beaker. The lipid from the aqueous sample was

further extracted using chloroform. The solvent system was evaporated in a rotary evaporator at 30 °C. Finally, the lipids were used for production of FAME.

### 2.4. Preparation of FAME

The FAME was synthesized by transesterification method using immobilized lipase. The lipase enzyme was obtained from Hi-Media, India and its activity is 16 IU/mg. Before transesterification process the algal oil was heated to 60 °C for 30 min to reduce viscosity. The immobilized lipase was prepared by adding 1 mg of lipase powder dissolved in 1 mL of sterilized distilled water. Then the lipase enzyme was mixed with sodium alginate solution (2%), the mixer was dripped into cold sterile 0.2 mol/L CaCl<sub>2</sub> using sterile syringe from a constant distance and was cured at 4 °C for 1 h. The beads were hardened by suspended again in a fresh CaCl<sub>2</sub> solution for 24 h at 4 °C with gentle agitation. After immobilization, the beads were separated through filtration and washed with 25 mmol/L phosphate buffer (pH 6.0), in order to remove excess calcium chloride and enzyme. Then the beads were preserved using 0.9% NaCl solution for future use<sup>[18,19]</sup>. In a 20 mL screw cap vial, 5 mL of *N. oculata* oil was taken and methyl acetate was added (oil to methyl acetate molar ratio 1:12) along with 2 g of immobilised enzyme beads. The mixture was then agitated for 24 h, and centrifuged, transferred into separating funnel and left overnight. The upper layer containing FAME was transferred into a clean beaker and the content was washed with hot water until clear FAME was obtained. The mixture of FAME and water was centrifuged to remove water. Finally, the purified FAME was analysed by gas chromatography-mass spectrometer (GC-MS) and used for antimicrobial activity.

### 2.5. Microorganisms for antimicrobial studies

Strains of Gram-negative bacteria [*Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*)], Gram-positive bacteria [*Bacillus subtilis* (*B. subtilis*) and *Staphylococcus aureus* (*S. aureus*)] and yeast *Candida albicans* (*C. albicans*) were obtained from Department of Microbiology, Raja Muthiah Medical College and Hospital, Annamalai University, Tamilnadu, India. The bacterial stock cultures were maintained on nutrient agar slant at 4 °C. The selected bacteria and yeast were cultured (24 h) using peptone broth and Sabouraud's dextrose broth respectively for antimicrobial test.

### 2.6. Antimicrobial assay

*In vitro* antimicrobial assay was carried out using disc diffusion method<sup>[20]</sup>. About 20 mL of sterilized Muller-Hinton agar medium was poured onto sterilized Petri plates. After solidification, the test microbial suspensions were spread uniformly on the plates using a sterile cotton swab. The discs were prepared by using Whatman No. 1 filter paper approximately 5 mm in diameter and sterilized using an autoclave. Microalgal FAME was loaded onto sterile disc at different concentrations of 10, 20 and 30 µL/disc, air dried

and placed on the surface of the each plate. The positive controls for bacteria (streptomycin 10 µg/disc), for yeast (amphotericin B 100 IU/disc) and negative control (diluted methanol) were used. All the plates were incubated at 37 °C for 24 h. After incubation period, zone of inhibition was formed around the disc which was the evidence of antimicrobial activity[7].

### 2.7. DNA inhibition activity

The DNA inhibition activity was carried out according to Surendhiran *et al*[21]. Most sensitive microorganisms were selected for analyzing DNA inhibition. The fixed dosage was selected from antimicrobial assay. The FAME extracted was added to 5 mL nutrient broth containing bacterial culture and then this mixture was incubated at 37 °C in a shaker for 24 h. Culture without FAME extract was used as control. Then the DNA inhibition was analysed in molecular level by agarose gel electrophoresis, with a DNA sample volume of 20 µL.

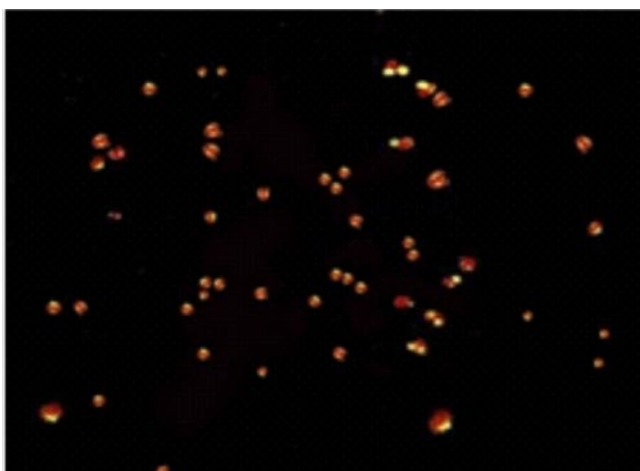
### 2.8. GC-MS analysis

Fatty acid composition of FAME produced from *N. oculata* FAMES were analysed by GC-MS (GC-MS-QP 2010, Shimadzu) equipped with VF-5 MS capillary column (30 mm length, 0.25 mm diameter and 0.25 µm film thickness). The column temperature of each run was started at 70 °C for 3 min, then raised to 300 °C and maintained at 300 °C for 9 min. GC conditions were: column oven temperature: 70 °C; injector temperature: 240 °C; injection mode: split; split ratio: 10; flow control mode: linear velocity; column flow: 1.51 mL/min; carrier gas: helium (99.9995% purity) and injection volume: 1 µL. MS conditions were: ion source temperature: 200 °C; interface temperature: 240 °C; scan range: 40–1000 m/z; solvent cut time: 5 min; MS start time: 5 min; end time: 35 min and ionization: EI (-70 eV) and scan speed: 2000 amu/second.

## 3. Results

### 3.1. Microscopic identification of intracellular lipid

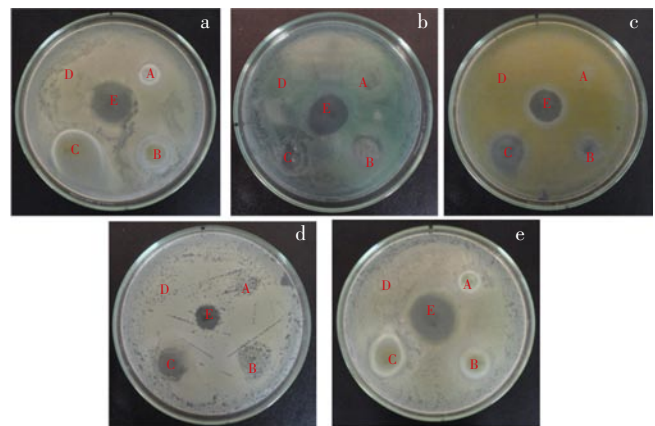
The intracellular lipid molecules were observed under fluorescent microscope at 100× with excitation at 450–490 nm and emission at 515 nm. Lipid molecules appeared as yellow dots, whereas cytoplasm was stained in red colour (Figure 1).



**Figure 1.** Nile red stained cells of *N. oculata* under fluorescent microscope.

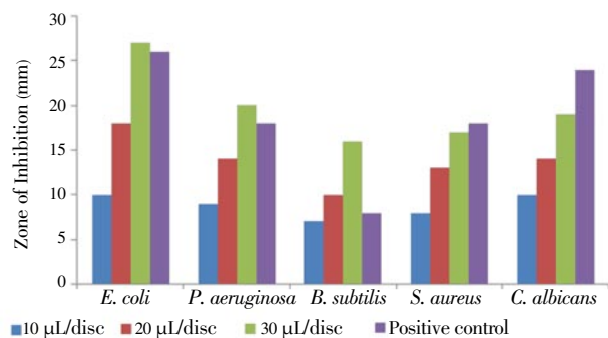
### 3.2. Antimicrobial activity of FAME

The antimicrobial activity of *N. oculata* FAME was tested against bacteria (both Gram-positive and Gram-negative) and yeast (*C. albicans*). In this study, we had found that FAME had the ability to inhibit both bacteria and yeast which was indicated by zone of inhibition around the disc (Figure 2). With three different concentrations of FAME (10, 20 and 30 µL/disc), the maximum concentration of 30 µL/disc resulted in maximum inhibition activity of all tested strains. Among the different microbial strains tested, *E. coli* and *P. aeruginosa* were found to be more sensitive with the zone of inhibition of 27 mm and 20 mm (Figure 2a and 2b) respectively than other microorganisms such as *B. subtilis* (16 mm), *S. aureus* (17 mm). They showed less susceptibility than the Gram-negative bacteria and higher susceptibility than the positive control (Figure 2c and 2d), whereas *C. albicans* (19 mm) showed moderate effect towards FAME of *N. oculata* than the Gram-negative bacteria (Figure 2e). Different FAME concentrations (µL/disc) and their respective zone of inhibition are shown in Figure 3.



**Figure 2.** Antimicrobial assay by disc diffusion method.

A: 10 µL/disc; B: 20 µL/disc; C: 30 µL/disc; D: Negative control; E: Positive control. a: *E. coli*; b: *P. aeruginosa*; c: *S. aureus*; d: *B. subtilis*; e: *C. albicans*.



**Figure 3.** Antimicrobial activity of FAME of *N. oculata* oil at different concentrations.

### 3.4. DNA inhibition activity

The most susceptible bacteria *P. aeruginosa* and *E. coli* towards FAME were selected for the DNA inhibition activity. After the agarose gel electrophoresis the DNA band was visualized under UV-transilluminator. DNA isolated from FAME treated bacterial culture was observed as small fragments and the control appeared as clear band (Figure 4). Formation of small fragments was due to the action of FAME of *N. oculata* on DNA of respective microorganisms and denature the DNA.



**Figure 4.** Illustration of sheared and normal DNA isolated from *P. aeruginosa* and *E. coli*.

A: Control DNA isolated from *P. aeruginosa* (untreated with FAME); B: Control DNA isolated from *E. coli* (untreated with FAME); C: Sheared DNA of *E. coli* isolated after FAME treatment; D: Sheared DNA of *P. aeruginosa* isolated after FAME treatment.

### 3.5. GC-MS analysis of FAME

By the GC-MS analysis the major fatty acid composition of FAME produced from *N. oculata* oil is shown in Table 1.

**Table 1**

Fatty acid profile of *N. oculata* oil.

Lipid number	Common name	Chemical name	Molecular structure	Fatty acid content (%)
C12:0	Lauric acid	Dodecanoic acid	C <sub>12</sub> H <sub>24</sub> O <sub>2</sub>	9.86
C16:0	Palmitic acid	Hexadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	19.39
C18:0	Stearic acid	Octadecanoic acid	C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>	10.76
C18:1	Oleic acid	9-Octadecenoic acid	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	35.22
C18:2	Linoleic acid	9,12-Octadecadienoic acid	C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>	8.15
C20:0	Arachidic acid	Eicosanoic acid	C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>	16.62

From the retention time, peak values of GC-MS result were analysed and observed. Lauric acid (C12:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and arachidic acid (C20:0), were commonly found in *N. oculata* (Table 1). From the data obtained from GC-MS, the presence of palmitic acid, oleic acid, linoleic acid and arachidic acid were the reasons for the antimicrobial activity[22–24].

## 4. Discussion

There are some studies on the antimicrobial activity of FAME. Agoramorthy *et al.* reported that FAME from leaves of blind-your-eye mangrove (*Excoecaria agallocha*) showed more activity against Gram-positive bacteria than the Gram-negative[22]. But in our study, Gram-negative bacteria were found to be more sensitive to FAME of *N. oculata* than Gram-positive bacteria. Similar results were also shown by Yuvaraj *et al.*, in which antibacterial activity of crude extracts were intense at minimum inhibitory concentration of seaweed *Cladophora glomerata*[25]. These results were due to the differences in the cell wall composition of Gram variables and permeability characteristics of different fatty acid molecules[22]. From the overall experiment, we inferred that *N. oculata* FAME possessed good inhibition effect against both types of bacteria than the commercial antibiotic streptomycin. This finding was in agreement with MubarakAli *et al.*[11]. But in case of *C. albicans* it was less

effective than the positive control amphotericin B.

In this present study, formation of small fragments was due to the action of FAME of *N. oculata* on DNA of respective microorganisms and denatured the DNA. This was in agreement with our previous study on DNA inhibition activity of genistein isolated from *Acalypha fruticosa* plant[21]. From these results we had concluded that FAME of *N. oculata* directly affected the DNA synthesis particular susceptible bacteria.

Many reports are available on antimicrobial activity of lipids or free fatty acids of marine macroalgae (seaweeds) but in this investigation, modification was done in the transesterification of *N. oculata* oil and their antimicrobial activity was increased[11]. Moreover, the large scale production of macroalgae bioactive compound synthesis is very difficult since controlled culture condition has to be maintained[26]. In case of microalgae, they can be cultivated easily under desirable condition and bioactive compounds could be synthesized for pharmaceutical.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgements

The authors thank the University for aiding with all facilities to carry out the research work. We also express our gratitude to the University Grants Commission, New Delhi, India for their financial support.

### Comments

#### Background

There are many reports related to antimicrobial activity of crude extracts of plants and marine macro and microalgae. Only a few studies are available on the inhibitory activity of FAME of marine microalgae and no report on molecular studies. Therefore it is very important to evaluate the antimicrobial activity of FAME from marine microalgae (*N. oculata* as well as other species).

#### Research frontiers

The present work deals with the use of FAME from microalgae as anti-bacterial and anti-candidal and evaluates the inhibition of DNA isolated from test pathogenic microorganisms. The authors showed that FAME from microalgae may have an inhibitory activity on both Gram-negative and positive bacteria and yeast (mainly Gram-negative bacteria) and these bioactive compounds could be synthesized for pharmaceutical purposes.

#### Related reports

There are many reports related to antimicrobial activity of crude extracts of marine macro and microalgae (Sethubathi

et al., 2010; Priyadharshini et al., 2012).

### Innovations and breakthroughs

Although there are some reports in the literature about the use of crude extracts of micro and macro algae, few data are available on the use of FAME from these organisms (mainly microalgae) as an inhibitory compound for bacteria growth. So, the information presented here suggests that microalgae could have a great potential in pharmaceutical industry.

### Applications

The present study indicates that FAME from microalgae could be efficiently used as bacteria growth inhibitor agent. In addition, the facility to maintain and produce microalgae in mass intensive or extensive cultures could propitiate the production of these compounds in the near future.

### Peer review

This research work presents an interesting set of data. The obtained results indicate that FAME have a great potential if used as inhibitor of bacteria growth.

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