J Appl Biotechnol Rep. 2022 June;9(2):603-613

Journal of
Applied Biotechnology Reports

doi 10.30491/JABR.2021.302307.1438

Original Article

Isolation and Purification of Antifungal Compounds from the Green Microalga *Chlorella vulgaris*

Yousef Y. Sultan [1], Diaa A. Marrez [1*]

[1] Food Toxicology and Contaminants Department, National Research Centre, Cairo, Egypt

Corresponding Author: Diaa A. Marrez, PhD, Assistant Professor, Food Toxicology and Contaminants Department, National Research Centre, Dokki, Cairo, Egypt. Tel: +20-1019621995, E-mail: diaamm80@hotmail.com

Received August 30, 2021; **Accepted** October 4, 2021; **Online Published** June 18, 2022

Abstract

Introduction: The current study aimed to purify antifungal compounds from *Chlorella vulgaris* extracts, fractions, sub-fractions and pure compounds against different strains of mycotoxigenic fungi.

Materials and Methods: Antifungal activity was conducted using disc diffusion assay, TLC-bioautography and Minimum Inhibitory Concentration (MIC). Isolation, purification and structure elucidation of antifungal compounds were carried out using column chromatography, Thin Layer Chromatography (TLC), UV-Vis spectrophotometer, Gas Chromatography–Mass Spectrometry (GC-MS), and Nuclear Magnetic Resonance (NMR).

Results: *C. vulgaris* Diethyl Ether Extract (DEE) showed the highest antifungal activity against all tested fungi with inhibition zone from 11.5 to 21.9 mm. By fractionation of DEE, Fraction F3 (chloroform:methanol, 50:50) and F5 (methanol 100%); sub-fraction CF3-10 and CF5-10 exhibited antifungal activity against all tested fungi. Two pure compounds, hydroxyphenophytin B and hexadecanoic acid methyl ester, with antifungal activity were isolated from CF3-10 and CF5-10, respectively.

Conclusions: *C. vulgaris* DEE and isolated compounds can be used as promising antifungal agents from natural sources against mycotoxigenic fungi at post-harvest or storage stages.

Keywords: *Chlorella Vulgaris*, Fractions, Antifungal, Mycotoxigenic Fungi, Bioautography

Citation: Sultan YY, Marrez DA. Isolation and purification of antifungal compounds from the green microalga *Chlorella vulgaris*. J Appl Biotechnol Rep. 2022;9(2):603-13. doi:10.30491/JABR.2021.302307.1438

Introduction

Chlorella vulgaris, freshwater unicellular green microalga, is characterized by its ability to grow under several nutritional modes (autotrophic, heterotrophic, and mixotrophic conditions).[1] Intensive algal biomass can be obtained using simple sources like CO_2 and sunlight and just small amounts of nutrients.[2] *Chlorella* has been proposed as a source of healthy supplementary food for human beings around the world.[3] It was also used for aquaculture and livestock feeding, pharmaceutical and cosmetics industries, biodiesel production, and waste water treatment by removing heavy metals and pesticides residues.[4-6]

Several studies demonstrated the health benefits of consuming *Chlorella* spp. extracts. They helped in reducing blood sugar levels, serum cholesterol levels, high blood pressure, ethionine intoxication, and in increasing hemoglobin concentrations. Their extracts were also used as hepatoprotective agents during malnu-trition and enhanced immune functions in humans.[7-9] Also, *Chlorella* spp. was used as a natural source of antitumor, antiviral, antioxidant, antibacterial and antifungal compounds in food supplements and as nutraceutical and food bio-preservative ingredients.[10-14]

Food can be contaminated at any point from farm to the end product. The majority of food spoilage caused by foodborne pathogenic bacteria and mycotoxigenic fungi cause foodborne diseases.[11,12] Mycotoxigenic fungi do not only cause food spoilage but can also produce mycotoxins, i.e., aflatoxins, ochratoxins, fumonisins, trichothecenes, zearalenone, and patulin that have an effect on human and animal health.[15] Several studies documented the use of plant and microalgae extracts as a natural source of food preservation.[16-23] *Chlorella vulgaris*, *C. minutissima* and *C. pyrenoidosa* were shown to have antimicrobial activity against wide ranges of foodborne microorganisms.[24,25]

Few studies have examined the antifungal activity of *C. vulgaris* extracts against mycotoxigenic fungi.[26,27] However, none of them purified these extracts to sub-fractions and pure compounds which can be used as bio-preservative in food against the most dangerous fungi.[28] So, the main objectives of this study were isolation, purification, structure elucidation of bioactive compounds from *C. vulgaris* against different strains of mycotoxigenic fungi.

Materials and Methods

Microalgae Cultivation and Extracts Preparation

Pure isolate of *C. vulgaris* was obtained from Marine Toxins Lab., National Research Centre, Egypt.[29] The culture media

used for cultivation of *C. vulgaris* was BG-11 medium.[30] At the stationary phase of growth (25 days), *C. vulgaris* biomass was harvested from 5 L flasks containing 3 L each and was dried overnight in a hot air oven at 50 °C (EHRET Labor-Und Pharmatechnik Gmbhund Co, Germany).

The dried *C. vulgaris* biomass (100 g) was extracted three times with different solvents of aqueous, ethanol, diethyl ether, ethyl acetate and hexane. The extracts were sonicated for 20 min using ultrasonic microtip probe of 400 watt (Ultrsonic Get 750, USA) and centrifuged at 3000 xg (Sigma Laborzentrifugen, Gmbh, Germany) for 10 min. Combined supernatants were evaporated to dryness at 40 °C using rotary evaporator (Laborota 4000, Heidolph, Germany) with an exception of aqueous extract which lyophilized using freeze dryer (Christ, ALPHA 1-4 LSC plus, Germany). Dried extracts were stored in labeled sterile vials in a refrigerator till further use.[24]

Antifungal Activity C. vulgaris Extracts

The lyophilized aqueous and other dried extracts were examined against mycotoxigenic fungi using disc diffusion technique.[16]

Tested Mycotoxigenic Fungi

Six fungal strains were used for antifungal assay, *Aspergillus flavus* NRRL 3357, *A. parasiticus* SSWT 2999, *A. carbonarius* ITAL 204, *A. ochraceus* ITAL 14, *Fusarium verticillioides* ITEM 10027 and *Penicillium verrucosum* BFE 500. The fungal strains were obtained from Applied Mycology Dept., Cranfield Unvi., UK. The stock cultures were grown on potato dextrose agar slant at 25 °C for five days and were then kept in a refrigerator till use.

Culture Media for Antifungal Assay

Yeast Extract Sucrose medium (YES), composed of 20 g yeast extract, 150 g sucrose, 20 g agar and 0.5 g $MgSO_4.7H_2O$ in 1000 ml distilled water was used for fungal disc diffusion test. Potato Dextrose Agar medium (PDA), composed of potato 200 g, dextrose 15 g and agar 20 g in 1000 ml distilled water and the pH was adjusted to 7.0, for determination of minimum inhibitory concentration.

Disc Diffusion Technique

The fungal strains were plated onto Potato Dextrose Agar (PDA) and incubated for five days at 25 °C. The spore suspension of each fungus was prepared in 0.01% Tween 80 solution. The concentration of spore suspension of each strain was adjusted by comparison with the 0.5 McFarland standard, the turbidity of the inoculum suspension represented approximately 2×10^8 cfu ml^{-1}. Petri dishes of YES medium were inoculated with 50 µl of each fungal culture and uniformly spread using sterile L- glass rod. Negative control was prepared by using DMSO and ceftriaxone (500 µg ml^{-1}) was used as a positive control. The inoculated plates were incubated at 25 °C for 48 h. Antifungal activity was evaluated by measuring the zone of inhibition (mm) against the tested fungus.[31,32]

Fractionations of C. vulgaris DEE Crude Extract

The DEE was chosen because of its high antifungal activity against the tested mycotoxigenic fungi. DEE was fractionated to fractions and sub-fractions using column chromatography.

Column Chromatography

The DEE was fractionated using column chromatography technique. Glass column (30 × 500 mm) was initially packed with 5 g of anhydrous sodium sulphate followed by 30 g of silica gel (0.06-0.2 mm, 70-230 mesh ASTM) using chloroform as a carrier solvent to create slurry. Finally, 5 g of anhydrous sodium sulphate was added to the top of silica gel to prevent column from drying. A portion of DEE (500 mg) in 10 ml chloroform was loaded to the column and allowed to flow at a rate of a drop sec^{-1}. The silica gel column was eluted with different mixtures (v/v) of chloroform: methanol (90:10, F1), (80:20, F2), (50:50, F3), (25:75, F4) and finally methanol 100% (F5) to give 5 fractions. The fractions, 50 ml each, were collected, evaporated under vacuum and stored for antifungal activity analysis. Fractions F3 and F5 were dissolved in chloroform:methanol (50:50) and methanol (100%), respectively and were passed through new prepared columns. Each fraction was divided into 10 sub-fractions (5 ml each).

Thin Layer Chromatography (TLC)

The TLC technique was performed on silica gel 60 aluminum, plate 10 × 10 cm (F254, Merck, Darmstadt, Germany). Sub-fractions were spotted 2 cm from the base of the plate and 1.5 cm intervals between spots. Fifty microliter of each active sub-fraction (20 mg ml^{-1}DEE) was spotted onto the silica gel plate and was dried for a few minutes. Afterwards, the plate was developed and run to 6 cm distance from the spotting base line with toluene: methanol: acetone:acetic acid (15:2:1:1) in a previously saturated glass chamber at room temperature. The developed plate was dried under normal air and the spots were read at 254 nm and 365 nm using UV chamber. The R_f (retention factor) values of the isolated compounds were determined by the following formula: R_f = Distance traveled by extract/distance traveled by solvent system.

Regarding the pure compounds isolated from the sub-fractions of DEE, their bands were scratched from TLC plates, dissolved in diethyl ether and filtrated to discard silica gel. The filtrate was dried at temperature below 40 °C using rotary evaporator. Its purity as a single spot was confirmed by re-chromatographed the dried filtrate on TLC with the same elution solvent system. To get high amounts of each pure compound, sub-fractions were spotted continuously

over the start base line of the TLC and scratched as mentioned before running the plates.

TLC Bioautography for Bioactivity Screening

Bioautographic evaluation was conducted in order to check the antimicrobial activity of the separated compounds on TLC plate. Previous developed TLC plates were sterilized by UV lamp for 30 min and were placed on 15 ml potato dextrose agar plate. Molten potato dextrose agar 20 ml seeded with 1 ml of spore suspension of each fungal strain was poured on a TLC plate as a second layer.[33] After solidification, the petri plates were kept at 4 °C for 3 h and incubated at 25 °C for 24-48 h. The inhibition zones were detected by staining with iodonitrotetrazolium chloride reagent 2% (INT). The inhibition zones appear as clear spots against the red background.[26] The inhibition zones were compared with the R_f values of untreated TLC plate contained compounds. Active bands were scratched from several TLC plates, dissolved in diethyl ether and filtrated to discard silica gel. Its purity as a single spot was confirmed by re-chromatographed on TLC with the same elution solvent system to verify its purity as a single compound.

Bioactive Compounds Identification and Structure Elucidation

GC-MS Analysis

The GC/MS analysis was performed using a Thermo Scientific, Trace GC Ultra/ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30 m, 0.25 mm, 0.1 mm film thickness). For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as the carrier gas at a constant flow rate of 1ml min^{-1}. The injector and MS transfer line temperature was set at 280 °C. The oven temperature was programmed at an initial temperature 50 °C (hold 2 min) to150 °C at an increasing rate of 7 °C min^{-1}, then to 270 °C at an increasing rate 5 °C min^{-1} (hold 2 min) then to 310 °C as a final temperature at an increasing rate of 3.5 °C min^{-1} (hold 10 min). The quantification of all the identified components was investigated using a percent relative peak area. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system.

Nuclear Magnetic Resonance (NMR)

NMR spectra were acquired in DMSO-d6 on a Jeol ECA 500 MHz NMR spectrometer, at 500 MHz. Standard pulse sequence and parameters were used to obtain one-dimensional ^{1}H and ^{13}C. ^{1}H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C NMR chemical shifts to DMSO-d6 and were converted to TMS scale by adding 29.8.

Determination of Minimum Inhibitory Concentration (MIC)

MIC against fungi was performed using the technique of Perrucci et al.[34] DEE crude extract, active fraction, sub-fractions and pure compounds at different concentrations were separately dissolved in 0.5 ml of 0.1% Tween 80 (Merck, Darmstadt, Germany), then mixed with 9.5 ml of melting, 45 °C, PDA and poured into Petri dish (6 cm). The prepared plates were centrally inoculated with 3 µl of fungal suspension. The plates were incubated at 25 °C for 24-48h. At the end of the incubation period, mycelial growth was monitored and MIC was determined.

Statistical Analysis

Statistical significance was determined using Statistica Version 9 (StateSoft, Tulsa, Okla., USA). In triplicates, the means of inhibition zones were determined by analysis of variance (ANOVA, one-way analysis) ($p<0.05$), followed by Fisher's LSD (Least significant differences) method ($\alpha = 0.05$) to compare significant differences between treatments.

Results and Discussion

Antifungal Activity of C. vulgaris Crude Extracts

The antifungal activity of *C. vulgaris* extracts against six strains of mycotoxigenic fungi is illustrated in Table 1. Significantly, diethyl ether extract exhibited the best inhibition among all extracts. *F. verticillioides* and *A. parasiticus* were the most affected fungi with 21.9 and 16.5 mm inhibition zones respectively. Ethyl acetate and hexane extracts also had antifungal activity against all tested fungal strains except *A. ochraceus* and *F. verticillioides*. Also, aqueous extract showed antifungal activity against all fungal species except *A. carbonarus* and *P. verrucosum*. This is while, ethanolic extract had activity against just *A. flavus* with inhibition zone 7.7 mm.

Many works examined *Chlorella* extracts against different fungi however none of them used mycotoxigenic strains which are able to produce mycotoxins in crops in case of availability of suitable conditions. Comparing with the present study, none of these studies reached a universe solvent against all tested fungi like DEE. For example, recently Dinev et al.[26] just examined the methanolic extract of *C. vulgaris* which exhibited activity just against *A. niger* among all tested fungi. Likewise, Vehapi et al.[27] used methanolic extract of *C. vulgaris* to control apple-infecting fungi which effectively inhibited *A. niger* more than the others. Also, Ghasemi et al.[24] found that *C. vulgaris* aqueous extract showed antifungal activity against *A. fumigatus* and *A. niger*, while hexane and methanolic extracts had no antifungal activity. Abedin and Taha[25] indicated that *C. pyrenoidos*a ethanolic, methanolic, acetone, and diethyl ether extracts had antifungal activity against *A. niger*, *A. flavus*, *F. moliliforme* and *P. herquei.* Also, Arun et al.[35] reported that acetone extract of *C. pyrenoidos*a had antifungal activity against *A. luchuensis*

and *F. oxysporum*, while methanolic extract showed antifungal activity only against *A. luchuensis*. In contrast, they found that *A. niger* was resistant against methanol, acetone and hexane extracts. Rajendran et al.[36] found that *Chlorella* sp. ethanol, acetone methanol, and chloroform extracts observed antifungal activity against *Fusarium* sp. Salem et al.[37] found that methanolic and acetone fractions of *C. vulgaris* showed antifungal activity against *F. oxysporum*.

Table 1. Antifungal Activity of *Chlorella vulgaris* Crude Extracts

Fungi	Inhibition Zone mm (Mean ± S.E)						
	-ve control	+ve control	Aqueous	Ethanol	DEE	Ethyl acetate	Hexane
A. flavus	0	16.1 ± 0.68^{a}	8.7 ± 1.25^{c}	7.7 ± 1.15^{d}	15.8 ± 2.25^{a}	14.3 ± 0.76^{b}	8.5 ± 0.50^{c}
A. parasiticus	0	11.8 ± 1.76^{b}	8.8 ± 0.76^{c}	0	16.5 ± 2.29^{a}	7.7 ± 0.58^{d}	8.3 ± 1.04^{c}
A. carbonarius	0	10.4 ± 0.36^{b}	0	0	11.5 ± 0.86^{a}	10.7 ± 1.04^{b}	7.3 ± 0.58^{c}
A. ochraceus	0	11.0 ± 0.50^{b}	8.7 ± 0.46^{c}	0	12.2 ± 1.25^{a}	0	0
F. verticilioides	0	11.2 ± 0.48^{c}	13.0 ± 0.86^{b}	0	21.9 ± 3.51^{a}	0	0
P. verrucosum	0	9.8 ± 0.76^{c}	0	0	15.8 ± 2.25^{a}	14.3 ± 0.76^{b}	8.5 ± 0.50^{d}

n = 3, S.E: standard error, 0: No inhibition, values with the same letter are not significantly different within raw at ($p \leq 0.05$),
Negative control: DMSO, Positive control: ceftriaxone.

Table 2. Antifungal Activity of *Chlorella vulgaris* DEE Fractions

Fungi	Inhibition Zone mm (Mean ± S.E)						
	-ve control	+ve control	F1	F2	F3	F4	F5
A. flavus	0	15.5 ± 0.50^{a}	8.0 ± 1.00^{b}	7.0 ± 0.28^{c}	8.0 ± 1.00^{b}	7.2 ± 0.76^{c}	8.3 ± 1.15^{b}
A. parasiticus	0	11.8 ± 0.86^{a}	8.0 ± 0.50^{c}	7.6 ± 1.15^{c}	7.7 ± 0.58^{c}	0	8.7 ± 1.08^{b}
A. carbonarius	0	11.5 ± 0.50^{a}	0	7.3 ± 0.58^{d}	7.0 ± 0.00^{d}	8.7 ± 0.58^{c}	9.7 ± 0.58^{b}
A. ochraceus	0	11.2 ± 0.96^{a}	0	8.3 ± 0.58^{b}	8.3 ± 0.58^{b}	11.2 ± 0.76^{a}	7.0 ± 0.00^{c}
F. verticilioides	0	11.5 ± 1.00^{a}	0	7.3 ± 0.58^{c}	7.7 ± 0.58^{bc}	8.0 ± 1.00^{b}	8.2 ± 0.76^{b}
P. verrucosum	0	9.9 ± 1.14^{a}	0	0	8.0 ± 1.00^{b}	7. 3 ± 0.58^{c}	7.0 ± 0.28^{c}

n = 3, S.E: standard error, 0: No inhibition, values with the same letter are not significantly different within raw at ($p \leq 0.05$).
Negative control: DMSO, Positive control: ceftriaxone.

Antifungal Activity of C. vulgaris Diethyl Ether Fractions

Table 2 represents the antifungal activity of *C. vulgaris* DEE fractions against the tested fungi. In general, *A. flavus* was the most susceptible strain which was inhibited by all fractions. Fractions F3 and F5 were able to inhibit all strains. Although F4 had no effect on *A. parasiticus*, it showed the highest activity against *A. ochraceus* with an inhibition zone of 11.2 mm. The lowest activity was observed using F1 which inhibited only *A. flavus* and *A. parasiticus*.

Abedin and Taha[25] reported that the diethyl ether fraction from *C. pyrenoidosa* had antifungal activity against various human and plants pathogenic fungi, *A. flavus*, *P. herquei*, *F. moniliforme*, *A. brassicae*, *A. niger*, *Saccharomyces cerevisiae* and *C. albicans*. Arun et al. [35] revealed that methanolic and acetone fractions from *C. pyrenoidosa* had antifungal activity against *A. luchuensis* and *F. oxysporum* but no activity was observed against *A. niger*. Mudimu et al.[38] indicated that the methanolic fractions of *C. sorokiniana* showed antibacterial and antifungal activity against *B. subtilis*, *P. fluorescens*, *E. coli*, *S. cerevisiae* and no activity was found against *C. albicans*.

Antifungal Activity of C. vulgaris Diethyl Ether Sub-fractions

Sub-fractionation of DEE fraction was done to increase the chance of isolation of more pure bioactive compounds and remove sub-fractions that had not any bioactivity against the tested strains.

Table 3 shows the antifungal activity of *C. vulgaris* DEE fraction F3. Sub-fractions CF3-7, CF3-8, CF3-9 and CF3-10 showed antifungal activity against all tested fungi followed by CF3-2 and CF3-6 which had antifungal activity against all tested fungi except *A. carbonarus* and *P. verrucosum*, respectively. All sub-fractions observed antifungal activity against *A. ochraceus* and *F. verticelloides*, followed by *A. flavus* which was sensitive for all sub-fractions except CF3-1. The highest antifungal activity was recorded in CF3-10 against *A. ochraceus* and *A. flavus* with an inhibition zone of 10.7 and 10.0 mm, respectively.

Table 4 illustrates the antifungal activity of *C. vulgaris* DEE fraction F5. Sub-fractions CF5-5, CF5-8, CF5-9 and CF5-10 showed antifungal activity against all tested fungi. *P. verrucosum* was the most sensitive one among the tested fungi except CF5-1. In contrast, all tested fungi showed resistance against CF5-1. The highest antifungal activity was observed by CF5-2 against *A. parasiticus* and *F. verticilioides* with an inhibition zone of 9.7 and 9.3 mm, respectively. Regarding to this point, Ghasemi et al.[24] indicated that lipids fractions of *C. vulgaris* showed antifungal activity against *A. niger*, *A. fumigatus*, *C. kefyr* and *C. albicans*. Mason[39] reported that the methanolic fraction of *Chlorella* sp. possessed antimicrobial activity against *E. coli* and *C. albicans*. Rajendran et al.[36] found that the methanolic, ethanolic and chloroform fraction of *Chlorella* sp. strongly showed antifungal activity against *Fusarium* sp.

Table 3. Antifungal Activity of C. vulgaris DEE Fraction F3

Fungi	Inhibition Zone mm (Mean± S.E)											
	-ve control	+ve control	CF3-1	CF3-2	CF3-3	CF3-4	CF3-5	CF3-6	CF3-7	CF3-8	CF3-9	CF3-10
A. flavus	0	15.8 ± 0.48^{a}	0	8.0 ± 1.00^{d}	7.3 ± 0.58^{e}	8.0 ± 1.00^{d}	8.7 ± 0.58^{c}	9.7 ± 0.58^{b}	7.7 ± 0.58^{de}	8.0 ± 1.00^{d}	8.7 ± 1.15^{c}	10.0 ± 1.73^{b}
A. parasiticus	0	11.5 ± 0.50^{a}	0	8.7 ± 0.58^{c}	0	0	7.7 ± 1.15^{e}	7.3 ± 0.58^{f}	7.7 ± 0.58^{e}	8.3 ± 1.15^{d}	8.3 ± 0.58^{d}	9.3 ± 1.04^{b}
A. carbonarius	0	11.8 ± 1.14^{a}	0	0	7.3 ± 0.58^{e}	9.3 ± 1.52^{b}	0	7.7 ± 0.58^{d}	7.3 ± 0.58^{e}	7.7 ± 1.15^{d}	8.3 ± 1.15^{c}	7.0 ± 0.00^{e}
A. ochraceus	0	11.5 ± 1.00^{a}	7.7 ± 1.15^{e}	8.0 ± 1.00^{e}	8.0 ±1.00^{e}	7.7 ± 0.58^{e}	8.3 ± 1.15^{d}	9.0 ± 1.73^{c}	9.0 ± 1.73^{c}	8.0 ± 1.00^{e}	8.7 ± 1.52^{c}	10.7 ± 1.52^{b}
F. verticillioides	0	11.4 ± 0.86^{a}	8.3 ± 1.15^{d}	9.0 ± 1.00^{c}	7.7 ± 1.15^{d}	9.7 ± 1.15^{b}	9.0 ± 1.00^{c}	7.0 ± 0.00^{e}	8.0 ± 1.00^{d}	8.0 ± 1.00^{d}	8.0 ± 1.00^{d}	8.7 ± 1.15^{c}
P. verrucosum	0	10.2 ± 0.58^{a}	0	8.7 ± 0.58^{b}	0	0	0	0	8.3 ± 1.15^{c}	7.0 ± 0.00^{d}	7.0 ± 0.00^{d}	9.0 ± 1.00^{b}

n = 3, S.E: standard error, 0: No inhibition, values with the same letter within raw are not significantly different at (p≤0.05). Negative Control: DMSO, Positive Control: ceftriaxone.

Table 4. Antifungal Activity of *C. vulgaris* DEE Fraction CF5

Fungi	Inhibition Zone mm (Mean± S.E)											
	-ve control	+ve control	CF5-1	CF5-2	CF5-3	CF5-4	CF5-5	CF5-6	CF5-7	CF5-8	CF5-9	CF5-10
A. flavus	0	15.8 ± 0.48^{a}	0	8.0 ± 1.00^{b}	0	7.7 ± 1.15^{c}	8.3 ± 0.58^{b}	7.7 ± 0.58^{c}	0	8.3 ± 0.58^{b}	7.7 ± 1.15^{c}	7.3 ± 0.58^{d}
A. parasiticus	0	11.5 ± 0.50^{a}	0	9.7 ± 1.15^{b}	0	0	8.3 ± 0.58^{c}	7.7 ± 0.58^{c}	7.3 ± 0.58^{d}	8.3 ± 0.28^{c}	7.7 ± 1.15^{c}	7.7 ± 1.15^{c}
A. Carbonarius	0	11.8 ± 1.14^{a}	0	0	0	7.0 ± 0.00^{b}	7.0 ± 0.00^{b}	0	0	7.0 ± 0.00^{b}	7.3 ± 0.58^{b}	7.3 ± 0.58^{b}
A. ochraceus	0	11.5 ± 1.00^{a}	0	0	8.0 ± 0.00^{b}	7.0 ± 0.00^{c}	7.7 ± 1.15^{b}	0	0	7.3 ± 0.58^{c}	7.3 ± 0.28^{c}	7.3 ± 0.58^{c}
F. verticilioides	0	11.4 ± 0.86^{a}	0	9.3 ± 0.86^{b}	0	0	7.3 ± 0.58^{d}	8.0 ± 1.00^{c}	0	7.7 ± 0.58^{c}	7.3 ± 0.58^{d}	8.0 ± 1.00^{c}
P. verrucosum	0	10.2 ± 0.58^{a}	0	7.7 ± 0.58^{c}	7.0 ± 0.00^{d}	7.0 ± 0.00^{d}	8.3 ± 0.58^{b}	7.7 ±0.58^{c}	7.3 ± 0.58^{d}	7.7 ± 0.28^{c}	7.3 ± 0.58^{d}	7.7 ± 0.58^{c}

n = 3, S.E: standard error, 0: No inhibition, values with the same letter within raw are not significantly different at (p≤0.05). Negative Control: DMSO, Positive Control: ceftriaxone.

Table 5. Inhibition of Fungal Growth on Bioautographic TLC Plates by *C. vulgaris* Sub-fraction CF3-10

R_f	Inhibition Zone					
	A. flavus	***A. parasiticus***	***A. carbonarus***	***A. ochraceus***	***F. verticillioides***	***P. verrucosum***
0.34	--	--	--	--	--	--
0.44	--	--	--	--	--	--
0.46	++	+++	+++	+++	++	+++
0.49	++	++	+++	++	+++	+
0.51	+	++	++	--	+	--
0.58	--	--	--	--	--	--
0.71	--	--	--	--	--	--

--: No inhibition, (+): Inhibition zone 2-5 mm, (++): Inhibition zone 5-7 mm, (+++): Inhibition zone >7 mm

TLC Bioautography of the Bioactive Compounds in CF3-10 and CF5-10

Seven clear bands were isolated from sub-fraction CF3-10 with retention factor values (R_f) of 0.34, 0.44, 0.46, 0.49, 0.51, 0.58 and 0.71. Also, five clear bands were separated from CF5-10 using the same elution solvent system with R_f values of 0.48, 0.54, 0.58, 0.62 and 0.68.

Examples of separated bioactive bands on TLC and bioautography against fungal strains are illustrated in Figure 1. Table 5 represents TLC bioautography of *C. vulgaris* sub-fraction CF3-10 bands against six strains of fungi. The spots of R_f 0.46 and 0.49 were observed in clear zones of inhibition against such fungus followed by spot of R_f 0.51 which had activity against tested fungi with the exception of *A. ochraceus* and *P. verrucosum*. No antifungal activity was observed in bands of R_f values 0.34, 0.44, 0.58 and 0.71 against all tested fungi.

TLC bioautography of *C. vulgaris* sub-fraction CF5-10 bands against mycotoxigenic fungi are illustrated in Table 6. The bands of R_f values 0.54 and 0.58 showed clear inhibition zone against all tested fungi followed by 0.62 which showed antifungal activity against tested fungi except *A. parasiticus* and *A. ochraceus*. This is while, bands of R_f values 0.48 and 0.68 had no antifungal activity against all tested fungi. As mentioned before, no available studies sub-fractionated the *C. vulgaris* and elucidated the pure compound similar to the current study. However, Abd El-Raouf et al.[40] revealed that all polar fractions of *C. vulgaris* culture filtrate that fractionated with TLC showed antifungal activity against *C. albicans, A. flavus, A. fumigatus, A. niger, F. solani, F. moniliform, F. oxysporum* and *P. chrysogenum.* Similarly, Balamurugan et al.[41] indicated that chlorophyll a, chlorophyll b and carotene which isolated from *C. salina* had antimicrobial activity against *E. coli, Klebsiella* sp., *P. aeruginosa* and *A. niger*.

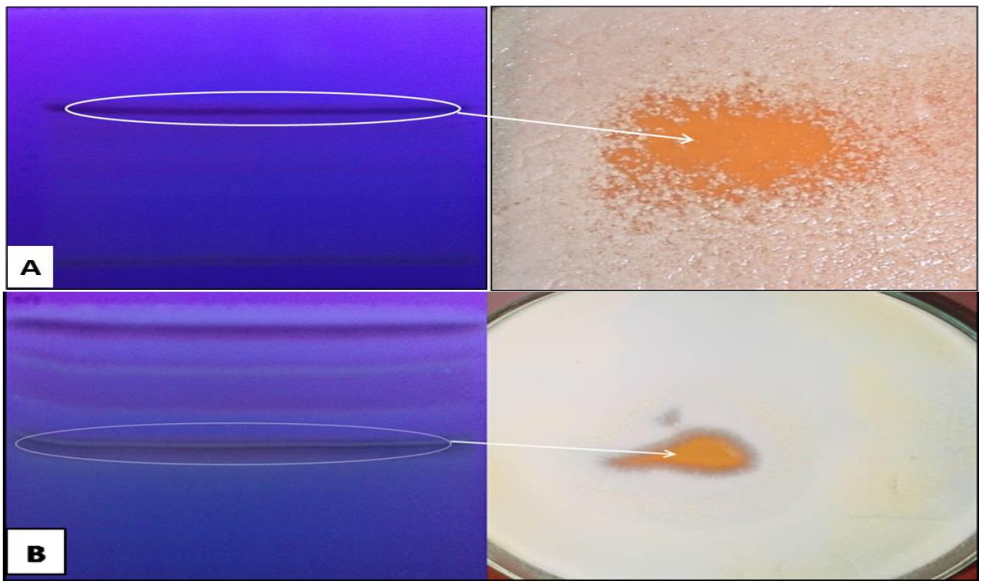


Figure 1. TLC and TLC Bioautography of *C. vulgaris* Sub-fractions; **A)** CF3-10 against *F. verticillioides,* **B)** CF5-10 against *A. ochraceus.*

Table 6. Inhibition of Fungal Growth on Bioautographic TLC Plates by *C. vulgaris* Sub-fraction CF5-10

R_f	Inhibition Zone					
	A. flavus	*A. parasiticus*	*A. carbonarus*	*A. ochraceus*	*F. verticillioides*	*P. verrucosum*
0.48	--	--	--	--	--	--
0.54	++	+	+++	+	++	++
0.58	+++	++	+++	++	+++	+++
0.62	+	--	+++	--	++	+++
0.68	--	--	--	--	--	--

--: No inhibition, (+): Inhibition zone 2-5 mm, (++): Inhibition zone 5-7 mm, (+++): Inhibition zone >7mm

Structure Elucidation of Isolated Bioactive Compounds from C. vulgaris Fractions

Spots had R_f 0.46 in sub-fraction CF3-10 and R_f 0.58 in CF5-10 showed the highest activity against all tested fungi. After conformation of the scratched band from different TLC plates of these compounds on TLC as pure compounds, they were coded CF3-10A (compound 1) and CF5-10B (compound 2), respectively.

Structure Elucidation of Compound 1

The dark-green pigment of compound 1 showed UV absorption maxima at 681, 624, 544, 510, 408 and 381 nm. Characteristic

color of alkaloids with Dragendorf reagent indicated that this compound belonged to a chlorophyll derivative.[42,43] The GC-MS spectrum analysis showed that the molecular ion peak was m/z = 888 $[M+1]^+$ and suggested its structure to be $C_{55}H_{76}N_4O_6$ as illustrated in Figure 2. For confirmation, NMR analysis was performed and its data showed similar identification to those published before this compound. The ^{1}H-NMR spectrum showed signal at δ7.15 (s, H-13), δ 8.42 (s, H-20), δ 9.55 (s, H-5) and δ9.57 (s, H-10) in addition to signals of phytyl group (Figure 2). Therefore, the structure of compound 1 was hydroxyphenophytin B (3-Phorbinepropanoic acid, 9-acetyl-14-ethyl-13,14-dihydro-21-(methoxy carbonyl)-4,8,13,18-tetramethyl-20-oxo-3,7,11,15-tetramethyl-2-hexa decenyl ester).

Hydroxyphenophytin B and its isomers were isolated from different plants, algae and photosynthetic bacteria.[44,45] Li et al.[46] reported that Hydroxyphenophytin B which isolated from the leaves of *Biden pilosa* had anti-inflammatory activity. ÓNeal et al.,[45] indicated that 2,3-dihydroprophyrin and its derivative including chlorophyll, pheophytin, pheophobide found in higher plants and even bacteria which had interesting properties in the development of new drugs in photodynamic therapy with strongly antimicrobial, anticancer and anti-inflammatory activities.

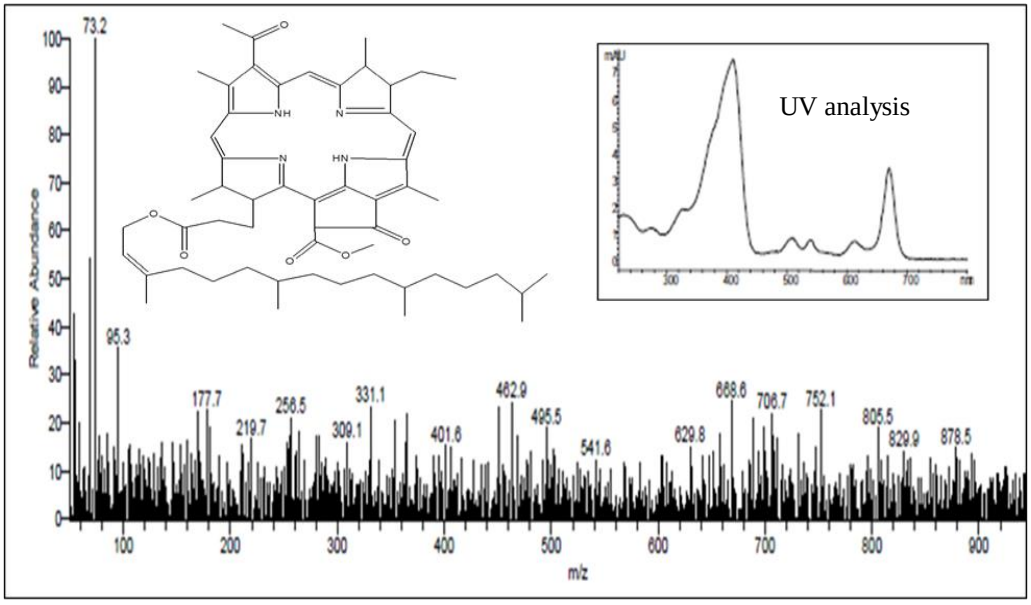


Figure 2. Chemical Structure, GC-MS Spectrum and UV Analysis of Hydroxyphenophytin B from *C. vulgaris*.

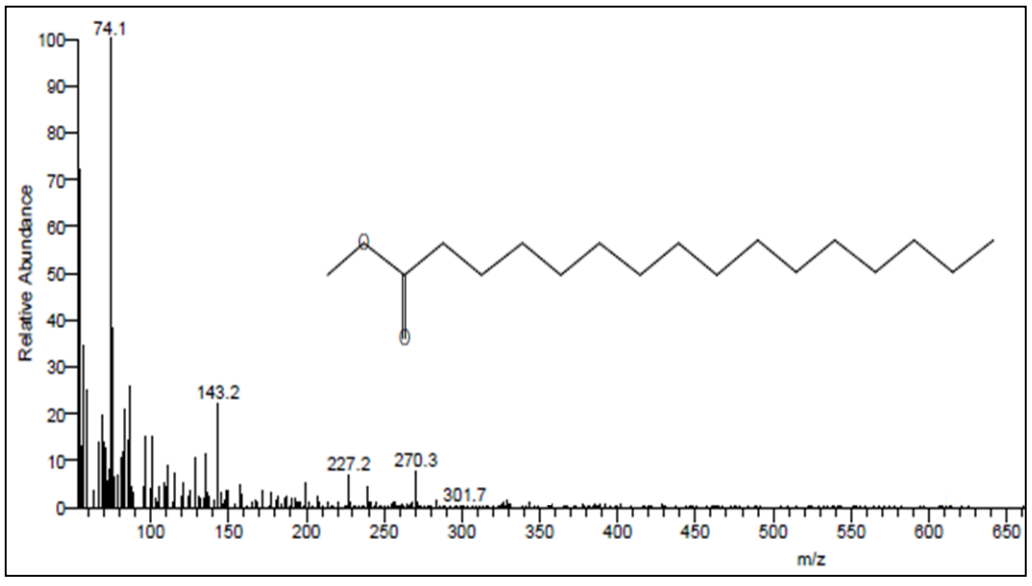


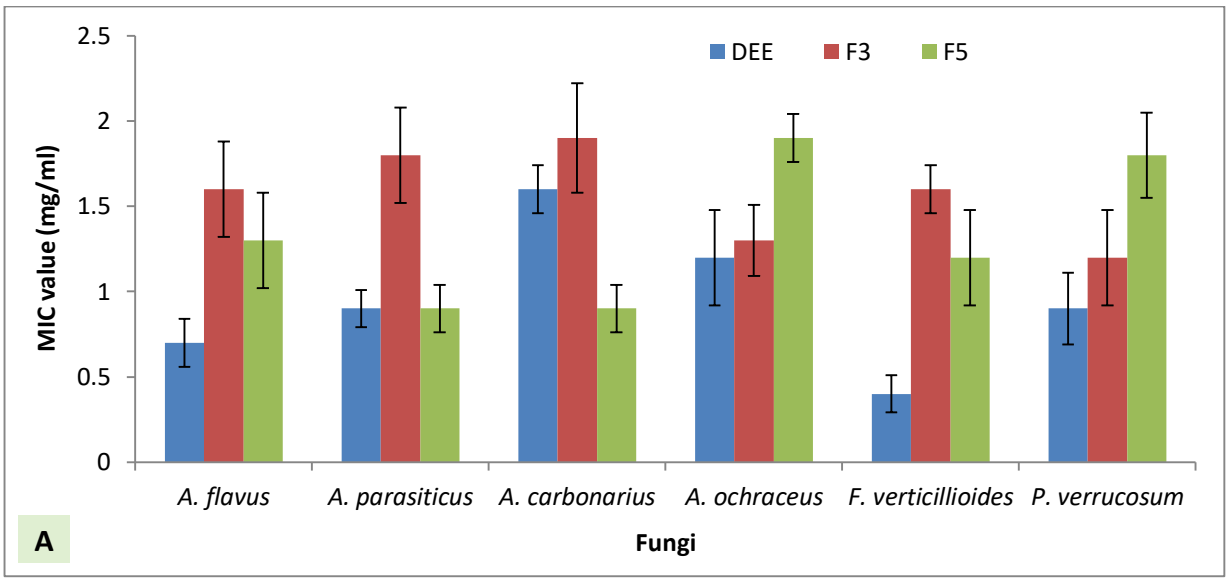
Figure 3. Chemical Structure and GC-MS Spectrum of Hexadecanoic Acid Methyl Ester.

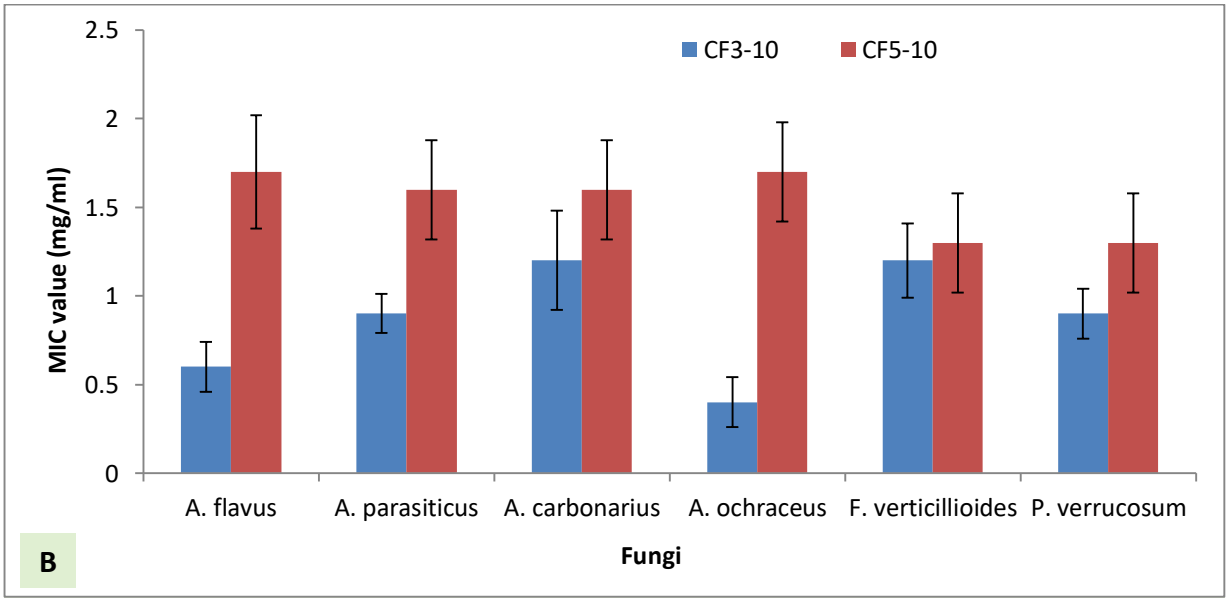
Structure Elucidation of Compound 2

Figure 3 illustrates the chemical structure of compound 2. It appeared after 26.98 min in GC-MS spectrum. Comparing with compound 1, it had small molecular weight (270 Dalton) and chemical structure $C_{17}H_{34}O_2$ typically as the structure identified by Abubacker and Deepalakshmi[47], Liu et al.[48] and He et al.[49] NMR analysis was performed to confirm the structure of compound 2. It gave signals with proton NMR (^{1}H-NMR) at δ = 2.4-2.7 ppm (2H, d), δ = 1.53 ppm (2H, m), δ = 1.2 ppm (26H, m) and δ = 3.54 ppm (3H, s). By the interpretation of its characterizations, the chemical structure referred to Hexadecanoic acid methyl ester.[50,51] There is no data about the purification of such compounds from *C. vulgaris*. However, it is isolated from different plants which exhibited antioxidant, anti-inflammatory, antibacterial, anticandidal, antifungal, and cancer preventive activities.[52-55] Moreover, methyl ester of hexadecanoic acid isolated from leaf extract of medicinal plant *Ruellia tuberosa* showed antifungal activity against *Penicillium* sp. and *Aspergillus* sp.[55]

MIC Values of C. vulgaris DEE, Fractions, Sub-fractions and Pure Compounds

The present research is considered to be the first study which has determined MIC as a sequence from crude extract to pure compound. MIC of DEE crude extract, fraction F3 and F5, sub-fraction CF3-10 and CF5-10 and pure compounds hydroxyphenophytin B and hexadecanoic acid methyl ester have been illustrated in Figure 4 (A-C). The highest activity of *C. vulgaris* DEE was recorded against *F. verticelloides* with MIC value of 0.4 mg ml^{-1}. Whereas, the lowest activity was showed against *A. carbonarus* with MIC values 1.6 mg ml^{-1}. *C. vulgaris* fractions showed highest activity using F5 against *A. parasiticus* and *A. carbonarus* with MIC 0.9 mg ml^{-1}, while the lowest activity was shown against *A. carbonarus*





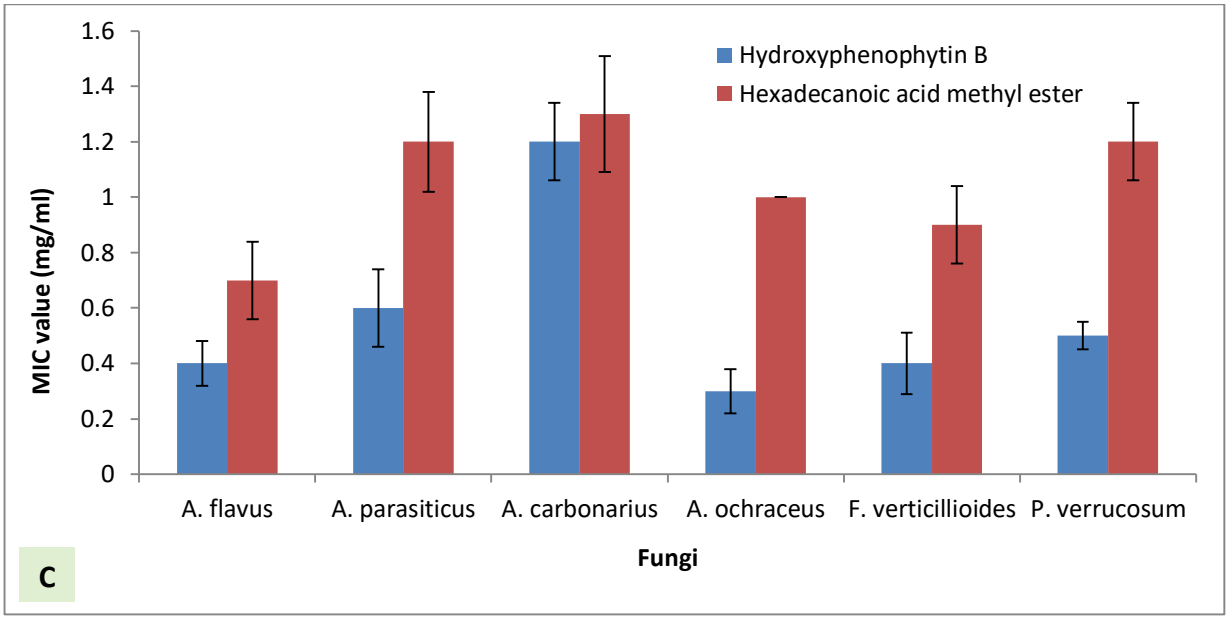


Figure 4. MIC Values (mg ml^{-1}) of *C. vulgaris*, **A)** DEE, Fractions F3 and F5; **B)** sub-fractions CF3-10 and CF5-10; **C)** Hydroxyphenophytin B and Hexadecanoic acid methyl ester, against tested fungi.

and *A. ochraceus* by F3 and F5, respectively with MIC value 1.9 mg ml^{-1}. Low concentration of *C. vulgaris* sub-fractions CF3-10 (0.4 mg ml^{-1}) was required to prevent the growth of *A. ochraceus*. However, high concentrations of CF5-10 was required to exhibit the inhibition effect on *A. flavus* and *A. ochraceus* with MIC values of 1.7 mg ml^{-1} (Figure 4B).

The MIC values of hydroxyphenophytin B (compound 1) against tested fungi ranged from 0.3 to 1.2 mg ml^{-1}. The highest bioactivity was recorded against *A. ochraceus* with MIC value of 0.3 mg ml^{-1} and the lowest activity was showed against *A. carbonarus* with MIC value of 1.2 mg ml^{-1}. The MIC values of Hexadecanoic acid methyl ester (compound 2) ranged from 0.7 to 1.3 mg ml^{-1}; the highest activity with MIC value of 0.7 mg ml^{-1} was recorded against *A. flavus*. This is while, the lowest activity with MIC value of 1.3 mg ml^{-1} was observed against *A. carbonarus* (Figure 4C). There are no available studies which have measured MIC of extracts, fractions, sub-fractions and pure compounds from *C. vulgaris*. However other algae were used. Jassbi et al.,[56] found that Hexadecanoic acid methyl ester isolated from a red algae *Hypneaflagelli formis* and two brown algae *Cystoseiramyrica* and *Sargassum boveanum* had antimicrobial activity against *E. coli*, *K. pneumonia*, *S. typhi*, *S. aureus*, *S. epidermidis*, *B. subtilis*, *A. niger* and *C. albicans* with MIC values ranging from 3 to 45.5µg ml^{-1}.

Conclusion

C. vulgaris, as an edible microalga, can be considered as a promising source of antifungal compounds especially against those producing mycotoxins in food. This can be used as an alternative agent to the chemical synthetic preservatives. DEE inhibited all tested fungi notply *F. verticillioides*, the causative source of maize spoilage. Two important compounds, hydroxyphenophytin B and hexadecanoic acid methyl ester, were purified and identified which is recommended to be used as a natural source of food preservatives.

Authors' Contributions

Conceptualization and original idea by YYS and DAM; Methodology by YYS and DAM; Writing-original draft preparation by DAM; Editing by YYS; All authors discussed the results and contributed to the final manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

Acknowledgment

This work was supported by the Science and Technology Development Fund (STDF) under grant No. 41535, and the National Research Centre, Cairo, Egypt. We thank our colleagues at this center for their valuable support.

References

1. Tomaselli L, Richmond A. Handbook of microalgal culture: biotechnology and applied phycology. Handbook of microalgal culture: biotechnology and applied phycology. 2004; pp. 146-147.
2. Liu J, Chen F. Biology and Industrial Applications of *Chlorella*: Advances and Prospects. in Microalgae Biotechnology, Posten C, Feng Chen S, Editors. Springer International Publishing: Cham. 2016; pp. 1-35. doi:10.1007/10_2014_286
3. Martins CF, Pestana JM, Alfaia CM, Costa M, Ribeiro DM, Coelho D, et al. Effects of *Chlorella vulgaris* as a feed ingredient on the quality and nutritional value of weaned piglets' meat. Foods. 2021;10(6):1155. doi:10.3390/foods10061155

4. Maliwat GC, Velasquez S, Robil JL, Chan M, Traifalgar RF, Tayamen M, et al. Growth and immune response of giant freshwater prawn *Macrobrachium rosenbergii* (De Man) postlarvae fed diets containing *Chlorella vulgaris* (Beijerinck). Aquac Res. 2017;48(4):1666-76. doi:10.1111/are.13004
5. Frumento D, Aliakbarian B, Casazza AA, Converti A, Al Arni S, da Silva MF. *Chlorella vulgaris* as a lipid source: Cultivation on air and seawater-simulating medium in a helicoidal photobioreactor. Biotechnol Prog. 2016;32: 279-84. doi:10.1002/btpr.2218
6. Marrez DA, Cieślak A, Gawad R, Ebeid HM, Chrenkova M, Gao M, et al. Effect of freshwater microalgae *Nannochloropsis limnetica* on the rumen fermentation *in vitro*. J Anim Feed Sci. 2017;26:359-64. doi:10.22358/jafs/81275/2017
7. Merchant RE, Andre CA. A review of recent clinical trials of the nutritional supplement *Chlorella pyrenoidosa* in the treatment of fibromyalgia, hypertension, and ulcerative colitis. Altern Ther Health Med. 2001;7(3):79-91.
8. Barrow C, Shahidi F. Marine nutraceuticals and functional foods. Press. 2008; pp. 367-403. doi:10.1201/9781420015812
9. Beheshtipour H, Mortazavian M, Mohammadi R, Sohrabvandi S, Khosravi-Darani K. Supplementation of *Spirulina platensis* and *Chlorella vulgaris* algae into probiotic fermented milks. Compr Rev Food Sci Food Saf. 2013;12:144-54. doi:10.1111/1541-4337.12004
10. Zielinski D, Fraczyk J, Debowski M, Zielinski M, Kaminski ZJ, Kregiel D, et al. Biological activity of hydrophilic extract of *Chlorella vulgaris* grown on post-fermentation leachate from a biogas plant supplied with stillage and maize silage. Molecules. 2020;25(8):1790. doi:10.3390/molecules25081790
11. Darwesh OM, Sultan YY, Seif MM, Marrez DA. Bio-evaluation of crustacean and fungal nano-chitosan for applying as food ingredient. Toxicol Rep. 2018;5:348-56. doi:10.1016/j.toxrep.2018.03.002
12. Marrez DA, Abdel-Rahman GN, Salem SH. Evaluation of *Pseudomonas fluorescens* Extracts as Biocontrol Agents Against some Foodborne Microorganisms. Jordan J Biol Sci. 2019;12(5):535-41.
13. Arabian D, Amiri P. Improving recovery process of omega-3 fatty acids from a native species of *Chlorella vulgaris* using integrated method. Appl Food Biotechnol. 2020;7(4):273-82. doi:10.22037/afb.v7i4.29779
14. Sedighi M, Jalili H, Ranaei S, Amrane A. Potential health effects of enzymatic protein hydrolysates from *Chlorella vulgaris.* Appl Food Biotechnol. 2016;3:160-69. doi:10.22037/afb.v3i3.11306
15. Hird S, Stien C, Kuchenmuller T, Meeting report: second annual meeting of the World Health Organization initiative to estimate the global burden of foodborne diseases. Intern J Food Microbiol. 2009;133:210-12. doi:10.1016/j.ijfoodmicro.2009.05.028
16. Marrez DA, Sultan YY. Antifungal activity of the cyanobacterium *Microcystis aeruginosa* against mycotoxigenic fungi. J Appl Pharm Sci. 2016;6(11):191-8. doi:10.7324/JAPS.2016.601130
17. Sultan YY, Ali MA, Darwesh OM, Embaby MA, Marrez DA. Influence of nitrogen source in culture media on antimicrobial activity of *Microcoleus lacustris* and *Oscillatoria rubescens*. Res J Pharm Biol Chem Sci. 2016;7(2):1444-52.
18. Marrez, DA, Sultan YY, Embaby MA. Biological activity of the cyanobacterium *Oscillatoria brevis* extracts as a source of nutraceutical and bio-preservative agents. Int J Pharmacol. 2017;13(8):1010-19. doi:10.3923/ijp.2017.1010.1019
19. Marrez DA, Naguib MM, Sultan YY, Higazy AM. Antimicrobial and anticancer activities of *Scenedesmus obliquus* metabolites. Heliyon. 2019;5(3):e01404. doi:10.1016/j.heliyon.2019.e01404
20. Marrez DA, Sultan YY, Naguib MM, Higazy AM. Antimicrobial Activity, Cytotoxicity and Chemical Constituents of the Freshwater Microalga *Oscillatoria princeps.* Biointerface Res Appl Chem. 2022, 12(1):961-77. doi:10.33263/BRIAC121.961977
21. Ordog V, Stirk WA, Lenobel R, Bancнřov6 M, Strnad M, Van Staden J, et al. Screening microalgae for some potentially useful agricultural and pharmaceutical secondary metabolites. J Appl Phycol. 2004;16(4):309-14. doi:10.1023/B:JAPH.0000047789.34883.aa
22. Khosravi K. The potential Health Benefits of Atgae and Micro Algae in Medicine: A Review on *Spirulina Platensis*. Curr Nutr Food Sci. 2011;7(4):279-85. doi:10.2174/157340111804586457
23. Hosseini S, Shahbazizadeh S, Khosravi-Darani K, Mozafari M. *Spirulina paltensis*: Food and Function. Curr Nutr Food Sci. 2013;9(3):189-93. doi:10.2174/1573401311309030003
24. Ghasemi Y, Moradian A, Mohagheghzadeh A, Shokravi S, Morowvat M. Antifungal and antibacterial activity of the microalgae collected from paddy fields of Iran: characterization of antimicrobial activity of *Chroococcus dispersus*. J Biol Sci. 2007;7(6):904-10. doi:10.3923/jbs.2007.904.910
25. Abedin RM, Taha HM. Antibacterial and antifungal activity of cyanobacteria and green microalgae. Evaluation of medium components by Plackett-Burman design for antimicrobial activity of *Spirulina platensis*. Glob j biotechnol biochem. 2008;3(1):22-31.
26. Dinev T, Tzanova M, Velichkova K, Dermendzhieva D, Beev G. Antifungal and antioxidant potential of methanolic extracts from *Acorus calamus* L., *Chlorella vulgaris Beijerinck, Lemna minuta Kunth* and *Scenedesmus dimorphus* (Turpin) Kutzing. Appl Sci. 2021;11(11):4745. doi:10.3390/app11114745
27. Vehapi M, Koзer AT, Yılmaz A, Ozcзimen D. Investigation of the antifungal effects of algal extracts on apple-infecting fungi. Arch Microbiol. 2020;202(3):455-71. doi:10.1007/s00203-019-01760-7
28. Sultan Y, Magan N. Mycotoxigenic fungi in peanuts from different geographic regions of Egypt. Mycotoxin Res. 2010;26(2):133-40. doi:10.1007/s12550-010-0048-5
29. Marrez DA, Naguib MM, Sultan YY, Daw ZY, Zaher SS, Higazy AM. Phytoplankton profile and toxicity assessment of dominant algal species from different Egyptian aquatic ecosystems. Res J Pharm Biol Chem Sci. 2016;7(2):1453-61.
30. Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. Microbiology. 1979;111(1):1-61. doi:10.1099/00221287-111-1-1
31. Chauhan A, Chauhan G, Gupta PC, Goyal P, Kaushik P. *In vitro* antibacterial evaluation of *Anabaena* sp. against several clinically significant microflora and HPTLC analysis of its active crude extracts. Indian J Pharm. 2010;42(2):105-7. doi:10.4103/0253-7613.64490
32. Medeiros MA, Oliveira DC, Rodrigues DD, Freitas DR. Prevalence and antimicrobial resistance of *Salmonella* in chicken carcasses at retail in 15 Brazilian cities. Rev. Panam Salud Public. 2011;30:555-60.
33. Hamburger MO, Cordell GA. A direct bioautographic TLC assay for compounds possessing antibacterial activity. J Nat Prod. 1987;50:19-22. doi:10.1021/np50049a003

34. Perrucci A, Okmen A, Gulluce M, Akpulat H, Dafera D. The in vitro antimicrobial and antioxidant activities of the essential oils and methanol extracts of endemic *Thymus spathulifolius*. Food Contl. 2004;15:627-34. doi:10.1021/jf035094l
35. Arun N, Gupta S, Singh DP. Antimicrobial and antioxidant property of commonly found microalgae *Spirulina platensis*, *Nostoc muscorum* and *Chlorella pyrenoidosa* against some pathogenic bacteria and fungi. Int J Pharm Sci Res. 2012;3(12):4866. doi:10.13040/IJPSR.0975-8232.3(12).4866-75
36. Rajendran N, Karpanai Selvan B, Sobana Piriya P, Logeswari V, Kathiresan E, Tamilselvi A, et al. Phytochemicals, antimicrobial and antioxidant screening from five different marine microalgae. J Chem Pharm Sci. 2014;7:78-85.
37. Salem OM, Hoballah EM, Ghazi SM, Hanna SN. Antimicrobial activity of microalgal extracts with special emphasize on *Nostoc* sp. Life Sci J. 2014;11(12):752-8.
38. Mudimu O, Rybalka N, Bauersachs T, Born J, Friedl T, Schulz R. Biotechnological screening of microalgal and cyanobacterial strains for biogas production and antibacterial and antifungal effects. Metabolites. 2014;4(2):373-93. doi:10.3390/metabo4020373
39. Mason R. *Chlorella* and *Spirulina*: Green supplements for balancing the body. Altern Complement Ther. 2001;7(3):161-5. doi:10.1089/107628001300303691
40. Abdel-Raouf N, Ibraheem I, Al-Enazi NF, Al-Othman MR. Biological control of *Fusarium oxysporum f.* sp *lycopersici* and *Rhizoctonia solani* by using of *Chlorella vulgaris* culture filtrate. World Res J Biosci. 2013;1(1):12-7.
41. Balamurugan G, Bipin AG, Prakash S, Karthikeyan G, Balaji R, Sathish KC, et al. Pigment producing capacity of saline tolerant microalgae *Chaetoceros calcitrans*, *Chlorella salina*, *Isochrysis galbana*, *Tetraselmis gracilis* and its antimicrobial activity: a comparative study. J Microbiol Biotechnol Res. 2013;3(1):1-7.
42. Walter E, Schreiber J, Zass E, Eschenmoser A. Bakteriochlorophyll aGg und Bakteriophaophytin aP in den photosynthetischen Reaktionszentren von *rhodospirillum rubrum* G-9+. Helvetica Chimica Acta. 1979;62(3):899-920. doi:10.1002/hlca.19790620329
43. Wu X, Liu Y, Sheng W, Sun J, Qin G. Chemical constituents of *Isatis indigotica*. J Planta Med. 1997;63:55-7. doi:10.1055/s-2006-957604
44. Lotjonen S, Hynninen PH. Carbon-13 NMR spectra of chlorophyll a, chlorophyll a′, pyrochlorophyll a and the corresponding pheophytins. Org Magn Reson. 1983;21(12):757-65. doi:10.1002/omr.1270211208
45. O'Neal WG, Roberts WP, Ghosh I, Wang H, Jacobi PA. Studies in Chlorin Chemistry. 3. A practical synthesis of C,D-Ring symmetric chlorins of potential utility in photodynamic therapy. J Organic Chem. 2006;71(9):3472-80. doi:10.1021/jo060041z
46. Li D, Yan S, Proksch P, Liang Z, Li Q, Xu J. Volatile metabolites profiling of a Chinese mangrove endophytic Pestalotiopsis sp. strain. Afr J Biotechnol. 2013;12(24):3802-6.
47. Abubacker M, Deepalakshmi T. *In vitro* antifungal potential of bioactive compound methyl ester of hexadecanoic acid isolated from *Annona muricata* linn (Annonaceae) leaves. Biosci Biotechnol Res Asia. 2013;10(2):879-84. doi:10.13005/bbra/1211
48. Liu J, Chen F. Biology and industrial applications of *Chlorella*: advances and prospects. J Microalgae biotechnol. 2014:1-35. doi:10.1007/10_2014_286
49. He F, Wang M, Gao M, Zhao M, Bai Y, Zhao C. Chemical composition and biological activities of *Gerbera anandria*. Molecules. 2014;19(4):4046-57. doi:10.3390/molecules19044046
50. Saxena M, Faridi U, Srivastava SK, Darokar MP, Mishra R, Pal A, et al. A cytotoxic and hepatoprotective agent from *Withania somnifera* and biological evaluation of its ester derivatives. Nat Prod Commun. 2007;2(7):775-8. doi:10.1177/1934578X0700200714
51. Oyugi DA, Ayorinde FO, Gugssa A, Allen A, Izevbigie EB, Eribo B, et al. Biological activity and mass spectrometric analysis of *Vernonia amygdalina* fractions. J Biosci Tech. 2011;2(3):287-304.
52. Yang J, Guo J, Yuan J. *In vitro* antioxidant properties of rutin. LWT Food Sci Technol. 2008;41(6):1060-6. doi:10.1016/j.lwt.2007.06.010
53. Prabhadevi V, Sahaya SS, Johnson M, Venkatramani B, Janakiraman N. Phytochemical studies on *Allamanda cathartica* L. using GC–MS. Asian Pac J Trop Biomed. 2012;2(2):S550-4. doi:10.1016/S2221-1691(12)60272-X
54. Mujeeb F, Bajpai P, Pathak N. Phytochemical evaluation, antimicrobial activity, and determination of bioactive components from leaves of *Aegle marmelos*. Biomed Res Int. 2014;2014:497606. doi:10.1155/2014/497606
55. Senthilkumar P, Sambath R, Vasantharaj S. Antimicrobial potential and screening of antimicrobial compounds of *Ruellia tuberose* using GC-MS. Int J Pharm Sci Rev Res. 2013;20(1):184-9.
56. Jassbi AR, Mohabati M, Eslami S, Sohrabipour J, Miri R. Biological activity and chemical constituents of red and brown algae from the Persian Gulf. Iran J Pharm Res. 2013;12(3):339-48