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Evaluation of microalgae and cyanobacteria as potential sources of antimicrobial compounds

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ABSTRACT

In recent decades, marine microorganisms have become known for their ability to produce a wide variety of secondary bioactive metabolites. Several compounds have been isolated from marine microorganisms for the development of novel bioactives for the food and pharmaceutical industries. In this study, a number of microalgae were evaluated for their antimicrobial activity against gram-positive and gram-negative bacteria, including food and plant pathogens, using various extraction techniques and antimicrobial assays. Disc diffusion and spot-on-lawn assays were conducted to confirm the antimicrobial activity. To measure the potency of the extracts, minimum inhibition concentrations (MIultCs) were measured. Three microalgae, namely *Isochrysis galbana*, *Scenedesmus* sp. NT8c, and *Chlorella* sp. FN1, showed strong inhibitory activity preferentially against gram-positive bacteria. These microalgal species were then selected for further purification and analysis, leading to compound identification. By using a mixture of different chromatography techniques gas chromatography–mass spectrometry (GC–MS) and high-performance liquid chromatography (HPLC) and ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS), we were able to separate and identify the dominant compounds that are responsible for the inhibitory activity. Additionally, nuclear magnetic resonance (NMR) was used to confirm the presence of these compounds. The dominant compounds that were identified and purified in the extracts are linoleic acid, oleic acid, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). These compounds are the potential candidates that inhibit the growth of gram-positive bacteria. This indicates the potential use of microalgae and their antimicrobial compounds as biocontrol agents against food and plant pathogens.

1. Introduction

In the last few decades, bacteria have increased their resistance to antibiotics, and they have become a major concern for public health because the antibiotics are losing their effectiveness at alarming rates due to the development of antibiotic-resistant microorganisms (Monnet et al., 1998, O'Neill 2016). Treating infectious diseases, especially diseases caused by resistant pathogens, is becoming one of the largest healthcare challenges worldwide. Resistance to antibiotics has emerged in major bacterial species; some of these species are now likely to be resistant to most/all available antimicrobials, creating an 'antibiotic resistance crisis' (Rossolini et al., 2014). By 2050, it is expected that the human cost of this crisis will reach 300 million premature deaths, with the worldwide economy losing $100 trillion as a result (Arias and Murray 2015). A major collaboration between the nations of the world and various branches of science is required to face this problem (Berendonk et al., 2015). Therefore, the World Health Organization (WHO) initiated a worldwide plan (Organization 2015) that many countries are following. However, the use of antibiotics in

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areas such as pharmaceuticals, agriculture, aquaculture, and manufacturing still affect the environment in critical ways (Topp et al., 2018). Moreover, the effort to discover new antimicrobial compounds in recent decades has been met with countless challenges and, in many cases, failure. In the golden years of antibiotic discovery, the process typically consisted of culture screening wastewater/soil-derived organisms for activity against other microorganisms. However, these discoveries proved cumbersome and were effectively exhausted by the 1970s due to the repeated identification of similar compounds.

To address this, attention has turned to alternative sources such as marine life, which provides a rich and diverse array of novel, biologically active compounds. Recently, marine organisms research has increased in research markets (Jena and Subudhi 2019). A number of chemically unique metabolites with different biological activity among marine origins, such as those from microalgae, have been isolated and developed as food and pharmaceutical products (Faulkner 2000, Schwartsmann et al., 2001). The use of microalgae as an alternative source of antibiotics and preservatives has attracted similar attention. Eukaryotic and prokaryotic microalgae produce a wide range of biologically active compounds. These compounds include toxins, algaecides, plant-growth regulators and, most importantly, those with uses in the food and pharmaceutical industries (Żak et al., 2015, Bhattacharjee 2016).

Currently, microalgae and cyanobacteria are receiving a high level of attention from manufacturers and researchers alike. Based on the widely biodiverse properties of these microorganisms, they are expected to be strong commercial sources of high-value bioactive compounds. In particular, the antimicrobial properties of microalgae and cyanobacteria have been examined for their potential use as pharmaceutical antimicrobial compounds and food preservatives (Mendiola et al., 2007).

Some of the antimicrobial activities of microalgae and cyanobacteria have been linked to unsaturated fatty acids. The first such antimicrobial compound, chlorellin, was isolated from *Chlorella* sp.; it is a mixture of fatty acids that inhibit the growth of both gram-positive and gram-negative bacteria (Pratt et al., 1944). Furthermore, eicosapentaenoic acid (EPA), hexadecatrienoic acid and palmitoleic acid isolated from *Phaeodactylum tricornutum* have been shown to possess antimicrobial activity against methicillin-resistant gram-positive *Staphylococcus aureus* (Benkendorff et al., 2005, Smith et al., 2010). Similarly, unsaturated fatty acids from *Scenedesmus intermedius*, *Chaetoceros muelleri*, *Haematococcus pluvialis*, *Chlorococcum* sp. and *Skeletonema costatum* were also found to have antimicrobial effects against a wide range of gram-positive and gram-negative bacteria. In addition, organic extracts obtained from *Euglena viridis* and *S. costatum* showed inhibitory activity against *Pseudomonas* sp. and *Listeria monocytogenes* (Das et al., 2005, Terekhova et al., 2009). Ethanolic extracts from *Isochrysis galbana* and *Dunaliella salina* were also found to be active against four different bacterial strains with an inhibitory concentration (IC_{50}) of 100, 80 μg/ml, respectively (Verma et al., 2016). *Coccomyxa onubensis* fatty acids extracts showed inhibition against a different range of gram-positive and gram-negative bacteria and fungi, with a lowest minimum inhibition concentration (MIC) of 305 and 106 μg/mL against *E.coli* and *P. mirabilis*, respectively (Navarro et al., 2017). Additionally, the pressurised liquid extracts of *Fucus vesiculosus* produced long-chain fatty acids that showed inhibition against *E. coli* (IC_{50} = 2.24 mg/mL) and *S. aureus* (IC_{50} = 1.27 mg/mL; (Otero et al., 2018). Another study found that *Rivularia mesenterica* ethanolic extract has a strong inhibitory effect against different antibiotic-resistant bacteria and fungi and the MIC ranged between 0.06 and 32.00 μg/mL (Skočibušić et al., 2019). Novel antimicrobial compounds have also been identified from microalgae and cyanobacteria; such a compound, EMTAHDCA, was isolated from *Nostoc* sp. and has a strong binding affinity to the protein of the targeted bacteria (Verma et al., 2016).

Microalgae and cyanobacteria have also shown antifungal and antiviral activity against a wide range of microorganisms. Ghasemi et al. (2007) reported that methanol and hexane extracts of *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Scenedesmus obliquus* and *Oocystic* sp. prevent the growth of *Aspergillus niger*, *Candida kefyr* and *Aspergillus fumigatus.* Karatungiols extracted from *Amphidinium* sp. are responsible for growth inhibition in *Aspergillus niger* and *Trichomonas foetus* (Washida et al., 2006). On the other hand, several studies have found virus growth-inhibitory compounds in microalgae. For instance, sulphated polysaccharide compounds isolated from *Navicula directa* and *Chlorella autotrophica* inhibit the replication of and hyaluronidase the enzymes in VHSV, ASFV, HSV1 and 2 and the influenza A virus (Fábregas et al., 1999, Lee et al., 2006). The aim of the present study was to purify, screen and evaluate different microalgal species for their ability to produce antimicrobial compounds.

2. Materials and methods

2.1. Chemicals

Chemicals were analytical or HPLC grade; they were purchased from Merck (Bayswater, VIC, Australia) and Sigma-Aldrich (Castle Hill, NSW, Australia). Bacterial growth discs were purchased from Edwards Group (Narellan, NSW, Australia).

2.2. Algal strains and growing conditions

Fourteen microalgal and two cyanobacterial strains from the Algae Biotechnology Laboratory collection (University of Queensland (UQ)) were screened for antimicrobial activity (Table 1). The microalgal strains that were used were *Pavlova lutheri*, *Chaetoceros muelleri*, local *Chlorella* sp. PH1, local *Chlorella* sp. FN1, *Chlorella vulgaris*, *Chlorella* sp. BR2, *Dunaliella tertiolecta*, *Haematococcus pluvialis*, *Isochrysis galbana*, *Nannochloropsis* sp. BR2, *Scenedesmus* sp. NT8c, *Tetraselmis astigmatica*, *Tetraselmis chuii* and *Tetraselmis suecica*. The cyanobacterial strains were *Limnothrix* sp. and *Spirulina* sp.

Microalgae were sub-cultured from master cultures in 2 L conical flasks with an f/2 medium (AlgaBoostTM F/2 2000x; Ausaqua Pty Ltd, Wallaroo, SA, Australia) for marine microalgae and Bold's basal medium (BBM) for freshwater microalgae (Stein 1973). The different microalgal species were cultured according to Alsenani et al. (2019). After algae growth reached the late exponential phase, cultures were transferred to 20 L bioreactors under the same conditions once the optical density (OD) at 440 nm reached 2.5. Then, they were transferred to the UQ Algae Energy Farm in Pinjarra Hills, Brisbane, Australia to test the reproducibility of the antimicrobial compounds on an industrial scale and in an outdoor environment. Cultures were grown outdoors in a 2 m closed photobioreactor comprising 200 L transparent bag towers with a 36 cm diameter, and the temperature varied between 23.6 °C during the daytime to 13.9 °C at night, with a 12/12 h light cycle. Cultures were harvested via centrifugation for 5 min at 3000*g*, then washed with Milli-Q water. Each harvested biomass was divided into two batches: the first batch was used directly for antimicrobial assays, while the second batch was freeze-dried and then stored at −80 °C before the extraction process. In addition, the supernatants were collected and stored at −20 °C to be tested for extracellular antimicrobial activity.

Table 1
List of algal strains studied.

Algal strain	Origin/source	GenBank	Water type/Media	Temperature °C
Pavlova lutheri	Queensland Sea Scallops Trading Pty Ltd (QSST)/UK	JQ423159	Marine/F/2	28
Chaetoceros muelleri	QSST, CSIRO, Tasmania (CS-176)/ Oceanic Institute, Hawaii, USA	JQ423153	Marine/F/2 + Si	28
local *Chlorella* sp. PH1	Brisbane River	NA	Brackish/ TP, TAP	28
local *Chlorella* sp. FN1	Brisbane River	MK488058.1	Brackish/ TP, TAP	28
Chlorella vulgaris	Brisbane River	NA	Marine/F/2	28
Chlorella sp. BR2	Brisbane River	JQ423156	Brackish/ TP, TAP	28
Dunaliella tertiolecta	CSIRO, Tasmania (CS-175/8)/	NA	Marine/F/2	28
Haematococcus pluvialis	CSIRO, Tasmania (CS-321/8)/	NA	Fresh/BBM	28
Isochrysis galbana	QSST/UK	JQ423157	Marine/F/2	28
Nannochloropsis sp. BR2	Brisbane River	JQ423160	Brackish/F/2	28
Scenedesmus sp. NT8c	Northern Territory	KF286271.1	Fresh/BBM	28
Tetraselmis astigmatica	Maroochydore, Australia	NA	Marine/F/2	28
Tetraselmis chuii	QSST/CSIRO, Tasmania (CS-26)/ East Lagoon, Galveston, TX, USA	JQ423150	Marine/F/2	28
Tetraselmis suecica	QSST/CSIRO, Tasmania (CS-187)/ Brest, France	JQ423151	Marine/F/2	28
Limnothrix sp.	Brisbane River	NA	Marine/F/2	28
Spirulina sp.	CSIRO, Tasmania (CS-785/02/8)	NA	Fresh/BBM	28

NA: not applicable.

2.3. Extraction methods

Various extraction systems and methods were used to screen the bioactive compounds. The solvent systems used included ethyl acetate, acetonitrile, acetone, chloroform, methanol, ethanol, dichloromethane, dimethyl sulfoxide (DMSO), n-hexane, water, chloroform + methanol (2:1, v/v) and water + methanol (1:3, v/v). Method (a): 1 mL of each solvent was added to 10 mg of algal biomass (dry weight (DW)) in a porcelain mortar and the mixture was ground. The extracts were then transferred to 2 mL tubes and shaken for 90 min at room temperature. The tubes were centrifuged (5000*g*; 1 min), and the supernatants (i.e., intracellular extracts) were collected and transferred to another set of 2 mL tubes. Method (b): 10 mL of each solvent was added to 1 g DW of algal biomass and vortexed for 30 s before being incubated overnight in dark conditions to prevent light-sensitive compounds from degradation. After centrifugation, the extracts were transferred to new tubes and the solvents were completely evaporated via rotary evaporation. Then, the crude extracts were redissolved in 200 μL of the selected solvents. Method (c) involved subsequent extraction with a four-solvent system composed of water + methanol (1:3, v/v), chloroform, ethyl acetate and acetone, in that order. Each extraction step involved incubation overnight in dark conditions. The extract of each solvent was evaporated via rotary evaporation. The dried extracts were resuspended in 200 μL of the same solvent system. All extracts were stored at −20 °C and protected from direct light at all times.

The active crude extracts were fractionated using hexane, dichloromethane and methanol. Antimicrobial assays were then performed for each fraction, and the active fractions were taken for further analyses.

2.4. Target pathogens

Gram-positive human and plant pathogenic bacterial strains included *Listeria monocytogenes* (ATCC 7644), *Staphylococcus aureus* (ATCC 25925 and ATCC 9144), *Bacillus subtilis* (ATCC 6633 and ATCC 6051), *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Clavibacter michiganensis* and the gram-negative bacteria that were tested were *Escherichia coli* (ATCC 25922), *Salmonella typhoid* (ATCC 14028), *Pseudomonas syringae*, and *Proteus vulgaris*.

2.5. Antimicrobial assays

Both extra- and intracellular extracts of the selected microalgae and cyanobacteria were screened for their antimicrobial activity against the selected bacteria. The disc-diffusion method and the spot-on-lawn method were used to ensure the accuracy of the results. First, the disc-diffusion method was conducted by pouring tryptone soya agar (TSA) onto the plates and waiting for 20 min for solidification. Then the stock cultures were inoculated by spreading over the medium. Discs were loaded with 20 μL of the various microalgal extracts, allowed to dry, and then placed over the TSA using sterile forceps. After overnight incubation at 28 °C or 37 °C (depending on the bacteria), an inhibition zone was observed as a sign of antimicrobial activity. Second, the spot-on-lawn method was conducted by adding 10 μL of the selected bacteria to 7 mL of 0.7% TSA medium and pouring it on top of the solidified TSA plate. Then, 20 μL of various microalgal extracts were spotted onto the plates and allowed to dry for 45 min. Inhibition zones were observed after overnight incubation. Negative controls were used in each assay. The experiments were conducted in triplicate, and for each sample and the average diameter of the inhibition zone of each triplicate was calculated.

2.6. Minimum inhibition concentration (MIC)

The bacterium to be tested was streaked onto a tryptic soy agar plate and incubated at 28 °C or 37 °C for 24 h. One colony was then transferred to fresh tryptic soy broth (Difco, USA) (15 mL) and the cell density was adjusted to 10^4–10^5 colony-forming units (cfu)/mL. Test extracts were dissolved in 100% dimethyl sulfoxide (DMSO) and diluted with sterilised deionised water to 10% DMSO. The stock solution was diluted with 10% DMSO to give final concentrations of 1% DMSO. The extract concentrations were then serially diluted to 500, 250, 150, and 100 μg extracts/mL of 1% DMSO. Each dilution was transferred to a 96-well microtiter plate and freshly prepared microbial broth was added to each well. Negative growth controls contained only growth media, and positive growth controls contained growth media and bacterial suspension. The plates were incubated at 37 °C for 24 h, and the optical density of each well was measured spectrophotometrically at 600 nm (using POLARstar Omega Plate, BMG LABTECH, Offenburg, Germany). MIC values were determined after overnight incubation and recorded as the lowest concentration of each test solution that inhibited the growth of all the microorganisms in the wells. The experiments were conducted in triplicate for each sample and the average concentration of each triplicate was calculated.

2.7. GC–MS analysis

A Shimadzu GCMS-QP2010 ULTRA system was used to analyse and quantify the fatty acid content of each microalgal oil extract.

The analysis method was adopted from Chua et al. (2018) and Ma et al. (2018).

2.8. HPLC

Each active fraction was checked using a Shimadzu Nexera-i LC-2040C 3D analytical HPLC system equipped with a quaternary solvents delivery system, an online degasser, an autosampler, a column temperature of 22 °C and a photo-diode array detector (200–800 nm) coupled with an analytical workstation. Aliquots of the active fractions were redissolved in methanol, and 10 μL of these aliquots were injected into the HPLC system. Chromatographic separation was conducted on a Jupiter C4 microbore column (2 × 150 mm, 5-μm particle diameter, 100 Å pore size, Phenomenex, Belmont, CA, USA) using gradient elution consisting of 0.1% formic acid $_{(aq)}$ (A) and acetonitrile (B). The 110 min gradient was as follows: 0 min, 100% phase A; 90 min, 100% phase B; 110 min, 100% phase B. The flow rate was 0.2 mL/min. A 0.2 μm polytetrafluoroethylene (PTFE) membrane filter (Phenomenex, Torrance, CA, USA) was used to filter the samples before injection.

2.9. UHPLC-Q-TOF-MS analysis

Each fraction was analysed using an Agilent UHPLC-Q-TOF-MS system (Agilent Technologies, Santa Clara, CA, USA) to obtain the molecular weights of the components. The same elution gradient programme and solvents system as with the HPLC were used. The analysis method was adapted from Bose et al. (2015). Slight changes were made as follows: the sample volume was 100 μL, the capillary voltage was 2000 V, drying gas was 5.0 L min^{-1} and the fragmentor voltage was 150 V.

2.10. NMR analysis of crude extracts

Crude hexane extracts of active fractions were dissolved in approximately 1 mL of deuterated chloroform with 0.05% tetramethylsilane, transferred to a 5 mm NMR sample tube, and subjected for ^{1}H and ^{13}C NMR analysis in a 500 MHz Bruker Avance 500 high-resolution NMR spectrometer. For the ^{1}H NMR analysis, 32 scans were averaged, while for the ^{13}C NMR analysis, 1024 scans were averaged.

3. Results

3.1. Screening of microalgal extracts against human and plant pathogens

Among the various microalgae and cyanobacteria examined, three species, namely *I. galbana*, *Scenedesmus* sp. NT8c, and *Chlorella* sp. FN1, showed activity against gram-positive pathogens on plate growth inhibition assays (Fig. 1, Table 2). Different solvent extracts had different levels of antimicrobial activity against specific pathogens. Of all the extracts, the *I. galbana* ethanolic extract showed the highest level of antimicrobial activity against *L. monocytogenes*, though less activity was observed with the ethyl acetate extract when using a disc-diffusion assay. Moreover, using the spot-on-lawn method showed additional slight growth inhibition activity with the ethanol extract of *I. galbana* against *S. aureus*. However, no other extracts showed any activity when using the disc-diffusion method.

Using the spot-on-lawn method showed more growth inhibition activity by various extracts. Extracts of all three microalgal species showed a strong inhibition effect against all gram-positive bacteria (Fig. 1). Nevertheless, no antimicrobial activity was observed with all extracts against gram-negative pathogens. Also, both fresh microalgal biomass and supernatants showed negative results against all pathogens.

3.2. Minimum inhibition concentration (MIC)

The MICs of the extracts were measured using serial dilutions up to 100 μg of extracts /mL of 1% DMSO. The active growth inhibition concentrations for the microalgae tested ranged from 500 μg/mL to 1 mg/mL. The MIC for *I. galbana* was 500 μg/mL and was 1 mg/mL for *Chlorella* sp. FN1 and *Scenedesmus* sp. NT8c. There was no sign of growth inhibition below the mentioned concentrations of 100 μg/mL. The lowest concentrations that inhibited all bacterial growth and had clear solutions were identified and measured.

3.3. GC–MS analysis

In an attempt to identify the compounds responsible for the antimicrobial activity found in the microalgae, GC–MS was used to characterise the extracts. The GC–MS results showed various fatty acids methyl esters (FAME) contents and profiles for different species. The highest amount of α-linolenic acid as a FAME percent-

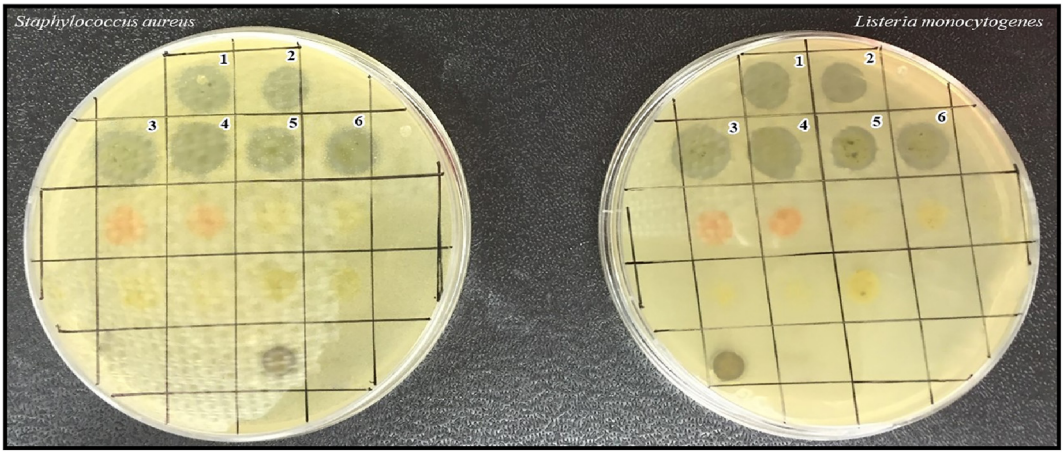

Fig. 1. Inhibition zones of *Staphylococcus aureus* and *Listeria monocytogenes* growth. 1: *I. galbana* crude extract; 2: *I. galbana* n-hexane fraction; 3: *Scenedesmus* sp. NT8c crude extract; 4: *Scenedesmus* sp. NT8c n-hexane fraction; 5: *Chlorella* sp. FN1 crude extract; 6: *Chlorella* sp. FN1 n-hexane fraction.

Table 2
Summary of antimicrobial activities of various species. Shown are mean values ± SD from three separately cultures.

Species		*I. galbana*		*Chlorella* sp. FN1	*Scenedesmus* sp. NT8c
Extracts		100% ethyl acetate	100% ethanol	70% methanol in water	70% methanol in water
Fractions			Hexane	Hexane	Hexane
Target pathogens	*L. monocytogenes*	12.67[a](±0.58)	19.67 (±0.58)*	16.67(±0.58)*	18.3(±1.15)*
	S. aureus	–	20(0)*	16.33(±0.58)*	17.67(±1.53)*
	B. subtilis	–	19.33(±0.58)*	15.67(±1.15)*	18.33(±1.15)*
	E. coli	–	–	–	–
	S. typhoid	–	–	–	–
	P. syringae	–	–	–	–
	C. michiganensis	–	16.33(±2.31)*	18.67(±1.53)*	17(0)*
	S. epidermidis	–	18.33(±0.58)*	16.67(±0.58)*	16.67(±0.58)*
	E. faecalis	–	19.33(±1.15)*	19.67(±0.58)*	18.33(±1.15)*

(–) No zone of inhibitions were observed.
[a] Mean diameter of triplicates inhibition zones were calculated.
* Values indicate statistically significant differences (*T*-test; $p < 0.05$).

Table 3
GC–MS identification of fatty acids methyl esters (FAME) detected in microalgal species, showing mean values ± SEs as mg/g dry weight from three separately grown cultures ($n = 3$).

Fatty acids		*I. galbana*	*Scenedesmus* sp. NT8c	*Chlorella* sp. FN1
Palmitic acid	C16:0	24.73 ± 1.07	21.73 ± 0.76	24.60 ± 1.02
Hexadecanoic acid	C16:1	10.64 ± 0.57	9.48 ± 0.54	9.16 ± 0.27
Octadecanoic acid	C18:0	0.86 ± 0.06	0.53 ± 0.04	0.58 ± 0.08
Oleic acid	C18:1	39.60 ± 2.02	4.25 ± 0.1	10.93 ± 0.39
Linoleic acid	C18:2n6	3.53 ± 0.04	6.36 ± 0.22	28.54 ± 0.96
α-Linolenic acid	C18:3n3	5.33 ± 0.46	28.57 ± 0.37	158.54 ± 3.38
EPA	C20:5n5	0.98 ± 0.13	n.d.	n.d.
DHA	C22:6n3	27.96 ± 2.73	n.d.	n.d.

n.d. – not detected.

age was found in *Chlorella* sp. FN1 (68.3%), while *I. galbana* had the highest amount of oleic acid (34.85%), and *Chlorella* sp. FN1 had the highest amount of linoleic acid (12.09%). Additionally, hexadecaenoic acid, oleic acid, linoleic acid and α-linolenic were detected in all three species in various concentrations while eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were found only in *I. galbana* (0.86% and 24.61%, respectively) as shown in Table 3 and Fig. 2.

3.4. HPLC analysis

The HPLC chromatograms of representative batches of crude *n*-hexane fractions of *I. galbana* and *Scenedesmus* sp. NT8c are shown in Fig. 3 A and B, respectively. The chromatograms show that most of the peaks appeared late in the run, after solvent B reached 50% (>50 min). The *I. galbana* chromatogram displayed two large peaks eluting about 70 and 86 min with concentrations of 13% and 32% of the total peak areas, while *Scenedesmus* NT8c showed multiple prominent peaks eluting at about 59, 66, 83 and 86 min with concentrations of 12%, 6%, 11% and 25%, respectively, of the total compounds and other relatively smaller peaks. The *n*-hexane fraction of the *Chlorella* sp. FN1 chromatogram showed a large peak eluting at about 54 min with a concentration of 41%, which most likely could be the one that produces the activity, while the other peaks were minor and of similar intensity (Fig. 3 C).

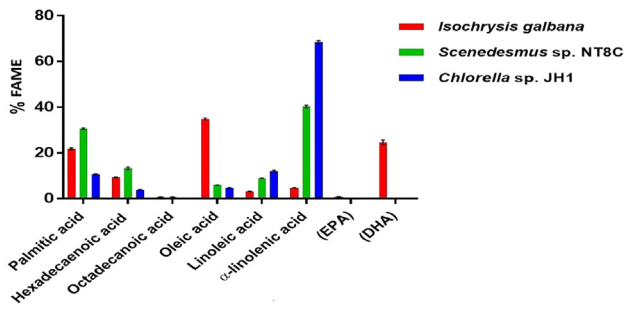

Fig. 2. Percentage of individual FAME detected in each microalgal species.

3.5. UHPLC-Q-TOF-MS analysis

The mass spectra were acquired for each component in the samples. Different mass spectra were measured with each fraction. Table 4 shows the molecular weights for each fraction. The mass spectra show the detected fatty acids that potentially mediate the growth-inhibition effect. Oleic acid is combined with stearic acid in a triglyceride form in all species. Additionally, pheophytin *a* also was detected in both *I. galbana* and *Chlorella* sp. FN1. Linolenic acid was detected in *I. galbana* as a form of triglyceride.

3.6. NMR analysis of crude extracts

^{1}H NMR analysis of the *n*-hexane extracts showed the presence of aliphatic $-CH_3$ and $—CH_2$ protons from the peaks in the range of 0.5–2 ppm. These protons could be attributed to the presence of lipids in the form of triglycerides or the methyl groups of carotenoids. The presence of carotenoids in the extracts was further verified by the peaks at around 5.4 ppm due to the =CH— protons, which are abundant in carotenoids. This set of peaks can also be attributed to the methylene protons of polyunsaturated fatty acids (Supplementary Fig. 1).

^{13}C NMR analysis confirmed the presence of lipids and carotenoids. Peaks in the range of 10–40 ppm were due to the alkyl carbon groups, which are the terminal and mid groups in fatty acids and carotenoids. Alkene carbons are present around 130 ppm, which is characteristic of polyunsaturated fatty acids and

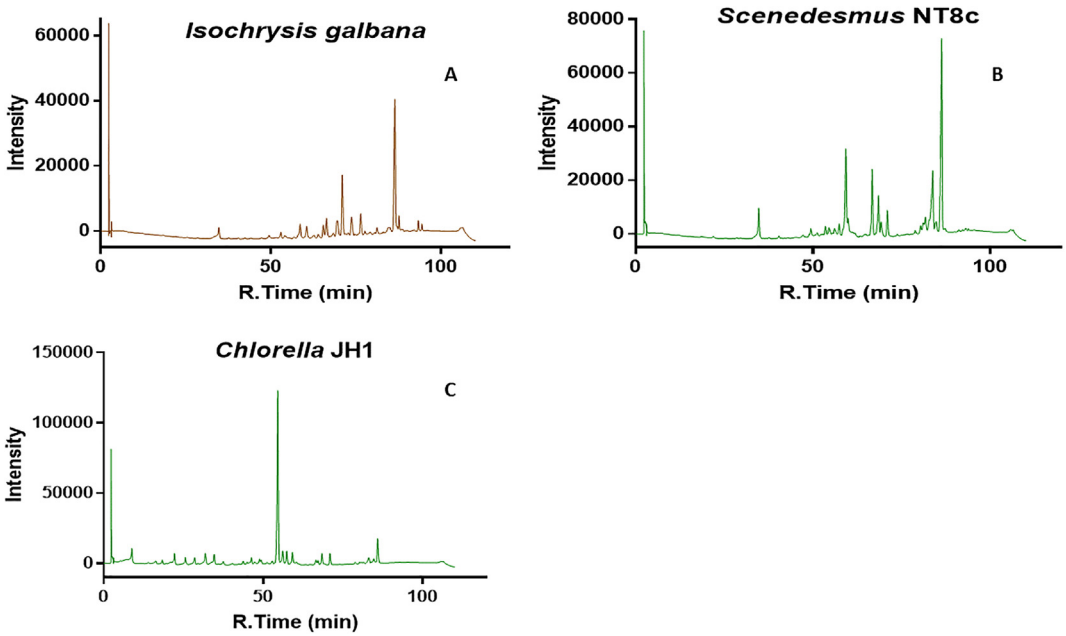

Fig. 3. HPLC chromatogram of the crude *n*-hexane fractions of the three microalgae tested.

Table 4
Summary of the detected compounds by UHPLC-Q-TOF-MS.

Species	Retention time (min)	M.W. (g/mol)	Compounds
I. galbana	61.9	872.6063	Trilinolenic glyceride
	71	887.5727	C18:0 + C18:1 + C18:1
	70	871.5713	pheophytin *a*
Scenedesmus	43	263.2006	α-Linolenol
sp. NT8c	43.9	284.3318	Stearic acid (C18:0)
	70	887.5691	C18:0 + C18:1 + C18:1
Chlorella sp.	62	587.4677	Hexadecanoic acid, 1, 1′-[1-
FN1			(chloromethyl)-1,2-ethanediyl]
	71	887.5682	ester
	72	871.5767	C18:0 + C18:1 + C18:1
			pheophytin *a*

carotenoids. The carbon attached to an alcohol is found in 50–52 ppm. This alcohol can be attributed to the alcohol groups attached to carotenoids like zeaxanthin in *I. galbana* or lutein in *Scenedesmus* sp. NT8c and *Chlorella* sp. FN1 or the alcohol groups from the glycerol backbone of mono- and diglycerides. Finally, the carbonyl group peaks of either carotenoids (e.g. fucoxanthin) or fatty acids are found in 180–190 ppm (Supplementary Fig. 2).

4. Discussion

There have been numerous studies of the antimicrobial activity of microalgae and cyanobacteria. The first antimicrobial compound discovered in microalgae was chlorellin, which was isolated from *Chlorella* sp. (Pratt et al., 1944). In addition, fatty acids, carotenoids, phycobiliproteins, and chlorophyll derivatives have been identified as key bioactive compounds in microalgae and cyanobacteria (Jørgensen, 1962; Bruce et al., 1967; Najdenski et al., 2013; Kasanah et al., 2019). Maadane et al. (2020) evaluated the antimicrobial activity of *I. galbana* and found that it had a strong inhibition effect on bacteria and fungi with a MIC that ranged between 2.6 and 4.3 mg/mL and activity linked to fatty acids and carotenoids contents In their study, the *n*-hexane fraction *I. galbana* also inhibited the growth of gram-positive bacteria. It has also been reported that the short and long-chain fatty acids from *H. pluvialis* and *Scenedesmus obliquus* are responsible for growth inhibition against *B. subtilis*, *S. aureus* and *E. coli* (Santoyo et al., 2009; Rodríguez-Meizoso et al., 2010; Catarina Guedes et al., 2011; Bashir et al., 2018). Surendhiran et al. (2014) reported that fatty acids from *Nannochloropsis oculata* showed activity against gram-positive and gram-negative bacteria. Moreover, Bruce et al. (1967) claimed that *I. galbana* had antimicrobial activity caused by pheophytin *a* and chlorophyllide *a* by showing a slight inhibition of *S. aureus* and strong inhibition of a wide range of gram-negative bacteria. These results were confirmed by Dvoretsky et al. (2019) who established that the inhibitive properties of *Chlorella* sp. were caused by fatty acids compounds. Mass spectrometry of the *Chlorella* sp. FN1 *n*-hexane fraction showed that it contained pheophytin *a*, which is consistent with Bruce et al. (1967) who detected the same compound in *I. galbana* and suggested that it was responsible for the antimicrobial activity. These results are also consistent with a study that performed antimicrobial screening on *Chlorella salina* and *I. galbana* and found that both had a strong inhibitory effect on pathogenic bacteria (Srinivasakumar and Rajashekhar, 2009).

In the current study, strong antimicrobial activity of various extracts has been observed against gram-positive bacteria. The strength of the activity was based on the minimum inhibition concentration (MIC) – the lowest concentration of extract that completely inhibits the growth of the bacteria compared with other extracts. The mechanism for antimicrobial activity of long-chain fatty acids (LC-FA) is not yet fully characterised, but it seems that interfering with the elongation process in the biosynthesis of bacterial fatty acids might be the inhibiting factor effect (Heath et al., 2001; Payne et al., 2001). In addition, it was claimed that the growth inhibition action due to the deleterious effect of fatty acids causes cell leakage of the bacterial cell membrane, or nutrient intake disruptions (Jüttner, 2001; Smith et al., 2010). The difference between gram-positive and gram-negative bacteria is the structure of the cell wall. The outer membrane of gram-negative species acts as an envelope. It is a system with multiple layers that include the outer membrane, the peptidoglycan cell wall, and the cytoplasmic membrane (the inner membrane) (Exner et al., 2017). This outer membrane works as a barrier to permeable compounds, and it excludes certain ones like antibiotics from

penetrating the cell (Silhavy et al., 2010). This function of the outer cell wall explains why gram-positive species were more susceptible and less resistant to antibiotics than gram-negative bacteria.

Following the initial profiling of LC-FAs in the active microalgae strains, oleic acid, DHA, palmitic acid, α-linolenic acid, and linoleic acid were detected. In several studies, linoleic acid and oleic acid have been proven to have antimicrobial activity from different sources (Dilika et al., 2000; Agoramoorthy et al., 2007; Kasanah et al., 2019). All the strains contained a high amount of palmitic acid, but saturated fatty acids showed little or no antimicrobial activity (Galbraith et al., 1971). To possess antimicrobial activity, the compounds must be water-soluble and remain sufficiently lipophilic so they can be adsorbed onto the cell surface, but this is not the case with saturated fatty acids. The presence of DHA in *I. galbana* might be the reason for the strong activity, along with linoleic acid and oleic acid, since DHA has inhibitory effects against gram-positive bacteria (Shin et al., 2007). Mass spectrometry and ^{1}H and ^{13}C NMR spectra have confirmed the presence of the measured fatty acids, which confirms the hypothesis of this study. Mass spectrometry detected oleic acid and linolenic acid in *I. galbana* and oleic acid in *Scenedesmus* sp. NT8c and *Chlorella* sp. FN1, which were measured before by GC–MS. In addition, the fatty acids mainly appeared in the form of triglycerides, while they are in the methyl ester form in GC–MS. Therefore, it is not necessarily the case that all the detected compounds will appear in both techniques.

The freshwater microalga *Scenedesmus* sp. has been shown to have notable antimicrobial properties against a wide range of bacterial pathogens. It was reported that acetone, methanol, and dichloromethane extracts slightly inhibited the growth of *Pseudomonas* sp. and *Xanthomonas oryzae*, as did hot- and cold-water extracts (Beena and Krishnika, 2011) Moreover, the antimicrobial activity of *Scenedesmus* NT8c extracts was in accordance with Marrez et al. (2019) who reported that different organic extracts have shown considerable inhibition against *S. aureus*, *E.coli*, and *S. typhi*. In the present study, the 70% methanol extract of *Scenedesmus* sp. NT8C showed strong inhibition against gram-positive bacteria but not gram-negative bacteria. This is partially consistent with other studies conducted on the same species (Table 3).

On the other hand, it has been claimed that linolenic acid inhibits enoyl-acyl carrier protein reductase (FabI) in bacteria which plays a fundamental role in the synthesis and growth of bacterial fatty acids (Zheng et al., 2005). High amounts of linolenic acid were found in *I. galbana*, *Scenedesmus* sp. NT8c and *Chlorella* sp. FN1. This might indicate that these species have antimicrobial properties. In all cases, the antimicrobial activity was produced by intracellular extracts. Thus, the antimicrobial compounds must be extracted by an efficient method. However, disc-diffusion assays did not show zones of inhibition, probably because the concentration of those compounds was very low or they had low diffusion rates through the discs. Therefore, the spot-on-lawn method was adopted to show more antimicrobial activity and to confirm the findings, as it enabled direct contact between the compounds and the pathogens.

This study confirmed that all three species are capable of producing antimicrobial compounds in both laboratory and industrial-scale conditions after being grown outdoors. The findings also showed that some microalgal species can produce antimicrobial compounds in a commercial setting or they can be developed as food supplements or as biopreservatives.

5. Conclusion

This study demonstrates the proof-of-concept that microalgae have the potential to generate potent antimicrobial compounds that act against human and plant pathogens when extraction processes are performed using appropriate solvents and conditions. The study found that three species of microalgae can produce strong antimicrobial compounds that inhibit the growth of different species of pathogenic gram-positive bacteria. The inhibition activity was caused by unsaturated fatty acids and carotenoids – compounds that were extracted efficiently from the selected species. These bacteriostatic or bactericidal bioactive compounds may act independently or through synergistic actions, although their precise mechanism of action is still unknown. Therefore further scrutiny and investigation are required to help unravel the mechanisms of microalgae–microbe interactions at play. In summary, this study has paved the way towards demonstrating the presence and viability of potential high-value pharmaceutical antimicrobials with the prospect of large-scale production.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary material

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