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Pressurized Fluid Extraction of Bioactive Compounds from *Phormidium* Species

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In the search for new functional ingredients with potential use in the food industry, extracts of unknown species of microalgae, such as *Phormidium* species have been studied. Three solvents of different polarities (i.e., hexane, ethanol, and water) have been used to obtain pressurized liquid extracts with different compositions. Moreover, extractions were performed at four different extraction temperatures (50, 100, 150, and 200 °C) with 20 min as extraction time. Antioxidant activity of the extracts has been measured by the TEAC assay. In general, hexane and ethanol extracts showed a higher antioxidant capacity that was mainly attributed to carotenoid compounds, as the TEAC value trend seems to be similar to the carotenoid content of the extracts. On the other hand, the high antioxidant activity of the 200 °C water extracts is likely related to the presence of Maillard reaction compounds produced by thermal degradation of the sample. β-Carotene, lutein, violaxanthin, and neoxanthin were identified in 150 °C ethanol extracts. Four different microbial species (*Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus niger*) were used to screen the potential antimicrobial activity of the *Phormidium* sp. extracts. The most sensitive microorganism was the yeast, *C. albicans*, whereas the fungus, *A. niger*, was the most resistant. In general, no drastic differences were found for solvents and temperatures tested, showing a very diverse nature of the compounds responsible for the antimicrobial activity of these microalgae. In ethanol extracts, antimicrobial activity could be mainly attributed to the presence of terpenes (i.e., β-ionone, neophytadiene) and fatty acids (i.e., palmitoleic and linoleic acids) in the samples. Toxicity studies carried out with the extracts evaluated in the present work showed a cellular toxicity lower than those of other cyanobacteria such as *Spirulina plantensis*.

KEYWORDS: Antimicrobial activity; antioxidant activity; *Phormidium* sp.; pressurized liquid extraction

INTRODUCTION

Algae are photosynthetic organisms with a great diversity of forms and sizes and can exist from unicellular microscopic organisms (microalgae) to multicellular organisms of great size (macroalgae). Some algae live in complex habitats submitted to extreme conditions (for example, changes of salinity, temperature, nutrients, UV–vis irradiation); therefore, they must adapt rapidly to the new environmental conditions to survive, producing a great variety of secondary metabolites biologically active to protect themselves, which cannot be found in other organisms (*1*). Also, considering their great taxonomic diversity, investigations related to the search for new biologically active compounds from algae can be seen as an almost unlimited field.

Microalgae such as cyanobacteria are well-known sources for products of commercial interest, such as proteins, fatty acids, vitamins, or pigments (*2*). They also produce a wide variety of secondary metabolites with different bioactivities (anti-inflammatory, antiviral, anticancer, antibacterial, cardioactive) (*3*). Cyanobacteria genera such as *Spirulina*, *Lyngbya*, *Nostoc*, and *Oscillatoria* have been extensively studied. Several works showed that *Spirulina*, or its extracts, can prevent or inhibit cancer and present immunopromoting effects and antimicrobial and antiviral activities (*4, 5*). Moreover, *Spirulina* contains a whole spectrum of natural mixed carotene and xanthophyll phytopigments that can be used as natural ingredients in the food industry, providing also some antioxidant activity (*6*). On the other hand, metabolites isolated from *Nostoc* exhibit potent cytotoxicity against human tumor cells lines (*7*) and also present

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antimicrobial and antifungal activity (*8*), whereas *Nostoc ellipsosporum* has been reported to possess a high antioxidant capacity (*9*). In the same way, biomolecules extracted from *Lyngbya* have been identified as potential lead compounds for the development of anticancer agents (*4*), whereas several works have demonstrated the antioxidant and antimicrobial activity of compounds extracted from *Oscillatoria* (*10*).

These data confirm the interest of the research focused on the search of bioactive compounds from cyanobacteria to be used, among other, to formulate functional foods, because there is a large collection of cyanobacterial strains still unexplored. In this respect, *Phormidium* sp. are filamentous cyanobacteria that remain rather uninvestigated, although some reports can be found in the literature concerning the antitumor and antiplasmodial activity of compounds isolated from different *Phormidium* strains (*11, 12*) and the inhibition of enzymatic activity of HIV-1 of some glycolipids isolated from *Phormidium tenue* (*13*). On the other hand, the *Phormidium* genus has been studied from a nutritional point of view, showing that it has a high nutritional value, containing around 50–63% proteins (DW) and a high calorie content: 32 kJ 10 g^{-1} of dry weight (DW) (*14*). It is, therefore, comparable to *Spirulina*, a cyanobacterium consumed as a protein source.

At present, studies directed toward the search for biologically active compounds use traditional extraction techniques with all of the known disadvantages (such as the use of large quantities of toxic organic solvents, long extraction times, low selectivity and/or low extraction yields, and exposure of the extracts to excessive heat, light, and oxygen). Pressurized liquid extraction (PLE) appears to be an advantageous alternative to the traditional processes as it uses less solvent in a shorter period of time, is automated, and involves retaining the sample in an oxygen-free and light-free environment (*15*). Whereas other environmentally friendly techniques, such as supercritical fluid extraction (SFE), are frequently used to obtain bioactive compounds from natural sources, PLE has not been widely applied as a routine tool in natural product extraction. However, recent studies have demonstrated the usefulness of PLE for the extraction of functional compounds from different matrices to be used as food ingredients (*16–18*).

Therefore, the goal of the present study was to obtain and characterize PLE extracts of different composition from *Phormidium* species and evaluate their potential as antioxidant and/or antimicrobial agents.

MATERIALS AND METHODS

Samples. *Phormidium* species (strain BNA 20_028) was obtained from the National Bank of Algae (Centre of Marine Biotechnology, Canary Islands, Spain) and grown photoautotrophically in BG11 medium (*19*). Cultures were bubbled with air, at 25 °C, and maintained with a circadian light/dark cycle (16:8) under a light intensity (cool-white lamps) of 80 μmol of photon m^{-2} s^{-1}. Cells in exponentially growing phase were used as inoculum for photobioreactor outdoor culture. Vertical tubular photobioreactors (140 L) with an air inlet at the bottom were used for outdoor cultures. Cells were grown in a semicontinuous culture, removing 50% of culture volume when maximal density was reached (about 0.5 g of DW/L). Biomass was harvested by filtration on a 25 μm sieve shaker, freeze-dried, and stored in an inert atmosphere until extraction.

Chemicals. 2,2-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and potassium persulfate were purchased from Sigma-Aldrich (St. Louis, MO), and carotene isomers mixture was from Merck (Darmstadt, Germany). 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and *all-trans*-β-carotene were from Fluka Chemie AG (Buchs, Switzerland). Ethanol was obtained from Scharlau Chemie S.A. (Barcelona, Spain). Hexane, light petroleum, acetone, and diethylamine were purchased from Panreac Quimica S.A. (Barcelona, Spain). Water was purified using a Milli-Q system (Millipore Corp., Billerica, MA). The sea sand that was used in the extraction cells was supplied by Panreac Quimica. Authentic violaxanthin, lutein, and neoxanthin standards isolated from phytoplankton were purchased from DHI Water and Environment (Hørsholm, Denmark).

Table 1. PLE Conditions (Solvent and Temperature; Extraction Time Constant and Equal to 20 min) of *Phormidium* Species Extractions, Yield Produced, and Antioxidant Activity (AA) Measured Using TEAC Assay

solvent	extraction temperature (°C)	yield (% DW)	AA[a]
hexane	50	0.47	0.547 ± 0.010
	100	1.68	0.506 ± 0.013
	150	4.30	0.349 ± 0.047
	200	6.54	0.353 ± 0.004
ethanol	50	5.28	0.269 ± 0.010
	100	8.48	0.318 ± 0.022
	150	10.62	0.335 ± 0.036
	200	40.91	0.267 ± 0.010
water	50	0.71	0.154 ± 0.005
	100	2.01	0.137 ± 0.003
	150	6.42	0.234 ± 0.006
	200	22.81	0.919 ± 0.016

[a] Antioxidant activity expressed as TEAC mmol of Trolox/g of extract.

Extraction Method. The samples were pretreated by freezing and mashing the microalgae with liquid nitrogen in a ceramic mortar. The process was repeated three times to induce cell-wall lysis. Extractions of *Phormidium* species were performed using an accelerated solvent extractor (ASE 200, Dionex Corp., Sunnyvale, CA) equipped with a solvent controller unit. **Table 1** shows the experimental conditions used with the three different solvents (i.e., water, ethanol, and hexane) employed for extraction. In the case of water extractions, it was necessary to place the sample into a filter paper box to avoid the agglomeration of the sample. Extractions were carried out in duplicate. Moreover, extractions were performed at four different extraction temperatures (50, 100, 150, and 200 °C) and 20 min as extraction time. First of all, an extraction cell heatup was carried out for a given time, which changed according to extraction temperature (the heatup time is automatically fixed by the equipment, that is, 5 min when the extraction temperature was 50 and 100 °C, 7 min at 150 °C, and 9 min at 200 °C). All extractions were performed in 11 mL extraction cells, containing 1.0 g of sample.

The extraction procedure was as follows: (i) sample is loaded in the cell; (ii) cell is filled with solvent to a pressure of 1500 psi, (iii) initial heatup time is applied; (iv) a static extraction with all systems valves closed is performed; (v) the cell is rinsed (with 60% cell volume using extraction solvent); (vi) solvent is purged from the cell with N_2 gas; and (vii) depressurization takes place. Between extractions, a rinse of the complete system was made to overcome any extract carry-over. To minimize the loss of volatiles and to avoid sample degradation, the extracts were quickly cooled to freezing temperatures by placing the vials in a water−ice bath. This was done for all of the extracts at the different temperatures tested.

Once cold, the extracts were subjected to solvent removal. For solvent evaporation, a Rotavapor R-200 (from Büchi Labortechnik AG, Flawil, Switzerland) was used for extracts obtained with organic solvents, and for water extracts, a freeze-dryer (Unitop 400 SL, Virtis, Gardiner, NY) was employed.

Antioxidant Analysis: TEAC Assay. The antioxidant activity of PLE extracts of *Phormidium* species was measured by the improved Trolox equivalent antioxidant capacity (TEAC) assay, performed essentially as previously described (*20*) for carotenoids standards. Briefly, $ABTS^{\bullet+}$ radical cation was generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate (final concentration) after incubation at room temperature for 16 h in the dark. The $ABTS^{\bullet+}$ radical solution was diluted with ethanol to give an absorbance of around 0.70 at 734 nm. The reaction was initiated by the addition of 10 μL of *Phormidium* sp. extract (dissolved in its respective solvent) to 0.990 mL of diluted

$ABTS^{\bullet+}$. The reactive mixture was allowed to stand until the reaction reached a steady state, and the absorbance was immediately recorded at 734 nm. Trolox was used as reference standard, and results were expressed as TEAC values (mmol of Trolox/g of extract). These values were obtained from at least three different concentrations of each extract tested in the assay giving a linear response between 20 and 80% of the blank absorbance. Moreover, all analyses were done in triplicate.

TLC. Three hundred micrograms of PLE extracts was applied on a 20 × 20 cm silica gel 60 F_{254} TLC plate (Merck). Plates were put in a closed chamber under dim light and eluted by 100 mL of petroleum ether/acetone/diethylamine mixture in the proportion 10:4:1 (v/v) as the mobile phase. Lutein and β-carotene were used as standards.

Antimicrobial Activity. *Microbial Strains.* The PLE extracts were individually tested against a panel of microorganisms including *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 11775, *Candida albicans* ATCC 60193, and *Aspergillus niger* ATCC 16404.

Bacterial strain stock cultures were kept on nutrient agar at 4 °C. *C. albicans* was kept on Sabouraud dextrose agar at 4 °C. *A. niger* spores were obtained in vitro from monoconidial cultures after incubation (7 days, 24 °C) on potato dextrose agar, harvested in sterile distilled water containing 0.1% Tween 80, and stored at 4 °C until used as inocula.

Determination of Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal and Fungicidal Concentration (MBC). A broth microdilution method was used, as recommended by National Committee for Clinical Laboratory Standards (NCCLS) (*21*), for determination of the MIC. All tests were performed in Mueller−Hinton broth supplemented with 0.5% Tween 20, with the exception of yeasts and fungi (Sabouraud dextrose broth + 0.5% Tween 20). The inocula of bacterial strains were prepared from overnight Mueller−Hinton broth cultures at 37 °C. Yeasts and fungi were cultured overnight at 25 °C in Sabouraud dextrose broth. Test strains were suspended in Mueller−Hinton (bacteria) or Sabouraud dextrose (yeast and fungi) broth to give a final density 10^7 cfu/mL. The *Phormidium* sp. extract dilutions in DMSO ranged from 250 to 10 mg/mL.

The 96-microwell plates were prepared by dispensing into each well 165 μL of culture broth, 5 μL of the inocula, and 30 μL of the different extract dilutions. The final volume of each well was 200 μL. Plates were incubated at 37 °C for 24 h for bacteria and at 24 °C for 48 h for yeast and fungus. Negative controls were prepared using 30 μL of DMSO, the solvent used to dissolve the microalgae extracts. Chloramphenicol and amphotericin B (Sigma, Madrid, Spain) were used as positive reference standards to determine the sensitivity of the microbial species used. After incubation, the MIC of each extract was determined by visual inspection of the well bottoms; bacterial growth was indicated by the presence of a white "pellet" on the well bottom. The lowest concentration of the extract that inhibited growth of the microorganism, as detected by the lack of the white pellet, was designated the MIC. The minimum bactericidal and fungicidal concentration was determined by making subcultures from the clear wells that did not show any growth. Each test was performed in triplicate and repeated twice.

Cytotoxicity Assay. The cytotoxic effect of the different extracts on Vero cells (African green monkey kidney cell line) (ATCC) was tested using the MTT assay, according to a published method (*22*). Monolayers of Vero cells in 24-multiwell plates were incubated with Eagle's Minimum Essential Medium (MEM) containing different concentrations of the extracts for 48 h at 37 °C. Cells were then washed with PBS, and 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) was added to each well and incubated for 4 h at 37 °C. Supernatants were discarded and formazan crystals dissolved in an extraction solution (10% sodium dodecyl sulfate in a mixture of dimethylformamide and water 1:1 v/v, adjusted to pH 4.7 with acetic acid) overnight at 37 °C. Formazan quantification was performed by measuring the optical density at 570 nm using a multiscanner autoreader with the extraction solution as a blank. The data were plotted as dose–response curves, from which the concentrationa required to reduce 50% the number of viable Vero cells (CC50) after 48 h of incubation with the different extracts were obtained.

HPLC-DAD Analysis. Analysis were performed with an HPLC Agilent HP 1100 series (Agilent, Palo Alto, CA) equipped with a diode array detector and an automatic Agilent 1200 Series injector.

Carotenoid compounds were analyzed with a YMC C30 (YMC, Schermbeck, Germany) C30 analytical column (5 μm, 250 × 4.6 mm i.d.). The mobile phase was a mixture of acetone and water as an isocratic mixture of 84% acetone and 16% water for the first 21 min, followed by a 4 min linear gradient to 97% acetone and 3% water for the reminder of the 50 min run. The flow rate was kept at 1 mL/min. Detection was accomplished by using a diode array system at a wavelength of 480 nm (scan from 190 to 600 nm) (*23*).The major carotenoids were identified by comparison of retention times and spectra against authentic standards (violaxanthin, lutein, neoxanthin, β-carotene).

GC-MS Analysis. Volatile compounds were analyzed with an Agilent-6890N GC system with a split/splitless injector coupled to an Agilent-5973N quadrupole mass spectrometer. The system was controlled by means of Agilent MSD Chemstation software. The column used was a 30 m × 0.25 mm i.d. fused silica capillary column coated with a 0.25 μm layer of SE-54 (HP-5MS, Agilent). The injection was carried out at 250 °C in split mode (ratio of 1:20). The volume of sample injected was 1 μL. Helium was the carrier gas (7 psi). The oven temperature was programmed as follows: 40 °C as the initial temperature (maintained for 2 min) to 150 °C in 24 min at 5 °C/min and from 150 °C to a final temperature of 300 at 15 °C/min.

Extracts were injected at different concentrations (4 mg/mL for the aqueous extract and 10 mg/mL for the ethanolic ones). All of the extracts were filtered with Millipore Millex HV, PVDF 0.45 μm filters prior to their injection.

A solvent delay of 4 min was selected before analysis of the compounds reaching MS. Compounds were tentatively identified by mass spectrometry in SCAN mode using a mass interval ranging from 35 to 450. Their spectra were compared with those in a mass spectrometry library (Wiley), with data found in the literature, and with standards when available. Additionally, to identify compounds more precisely, their linear retention indices were used when possible. Mixtures from *n*-undecane to *n*-octacosane (Aldrich, Sigma-Aldrich Chemie, Steinheim, Germany) dissolved in *n*-hexane were employed for linear retention index (RI) calculations.

RESULTS AND DISCUSSION

Pressurized Liquid Extraction. Because the possibility of extracting bioactive compounds from a natural source is directly related to their nature and to their solubility in the extracting solvent, three solvents (hexane, ethanol, and water) of different polarities were selected to evaluate their ability to extract antioxidant and/or antimicrobial compounds from *Phormidium* specie. Different extraction temperatures were also tested because, as has been demonstrated previously (*6, 23*) in work with microalgae, that temperature is the main variable involved in the PLE process (*23, 24*).

As mentioned, the extraction conditions employed as well as the resultant extraction yields are shown in **Table 1**. As can be observed, the higher extraction yield (expressed as percent dry weight of starting material) was obtained with ethanol as the extracting solvent, reaching, at high temperatures, around 40% yield. Moreover, the extraction yield increased with the extraction temperature, for all solvents used. This behavior can be explained by an increase of the solubility of the compounds with temperature along with an improved mass transfer from the sample to the pressurized solvent. The increase of the vapor pressure of the most volatile compounds with temperature could also enhance the extraction yield for some compounds.

To verify the chemical composition of the extracts and to determine if the differences among solvents and temperatures were qualitative or only quantitative, an analytical TLC was performed of all extracts (**Figure 1**). As can be seen, similar compositions were obtained for ethanol and hexane extracts, which were mainly carotenoids and chlorophylls. Differences at various temperatures, for the same solvent, were mainly

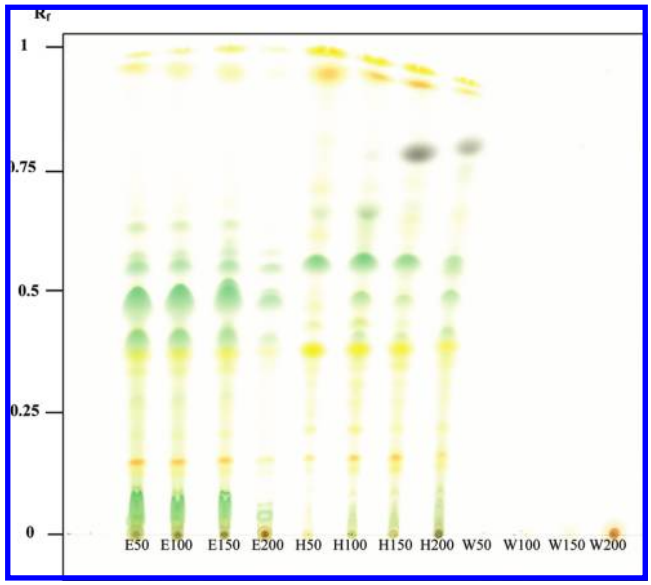

Figure 1. Analytical TLC of *Phormidium* sp. PLE extracts. E, ethanol; H, hexane; W, water. Standards: violaxanthin (R_f, 0.16); lutein (R_f, 0.36); β-carotene (R_f, 0.96); pheophytins (R_f, 0.78).

quantitative; for example, hexane extracts at 150 and 200 °C yielded pheophytins (gray band at $R_f = 0.78$) as degradation products of chlorophylls. On the other hand, as expected, no carotenoids or chlorophylls were observed for water samples. Interestingly, water extract at 200 °C provides a very high yield (>20%), but compounds of this extract were mainly polar, as can be deduced from the TLC (the spot did not move from the application point). Thus, compounds extracted with subcritical water at 200 °C were mainly odoriferous, colored polar compounds, with a characteristic smell.

Further analysis of this sample by HPLC with a method developed for polar compound analysis (data not shown) did not provide additional information on the composition or chemical nature because all of the compounds eluted did not show any identifiable spectra. Considering the composition of the microalgae in terms of amino acids and sugars, our hypothesis is that the compounds extracted could be Maillard reaction products produced by thermal degradation of the sample as this process has been described in high water activity food samples (*25*). Further studies are being conducted in our laboratory to confirm this hypothesis.

Antioxidant Activity of the PLE Extracts. Antioxidant activity of PLE extracts from *Phormidium* species was measured by TEAC assay (**Table 1**). The main advantage of this method is focused on wavelength absorption maximum at 734 nm of $ABTS^{\bullet+}$. The spectrophotometric measure at 734 nm eliminates color interference with antioxidant compounds such as carotenoids.

As has been mentioned previously, a similar behavior about extraction yield was shown with the three different solvents. The higher the extraction temperature, the higher yield of the extracts was obtained. Nevertheless, a temperature increase caused a different behavior in relation to antioxidant activity depending on the solvent used in the extraction. With regard to hexane extracts, antioxidant activity decreased as extraction temperature increased, being maximum at 50 °C. On the other hand, the antioxidant capacity of ethanol extracts was enhanced as the extraction temperature increased to 150 °C. Furthermore, a rise of temperature in water extractions caused only a slight effect on TEAC value, except for 200 °C. This extract showed a strong antioxidant activity, being the highest of all the extracts. In general, pressurized hexane extracts showed the highest TEAC values, except for water extract at 200 °C.

Antioxidant properties of *Phormidium* species have been focused on purification of their phycocyanins (*10*) as they have been described as potent antioxidants with several biological properties (*26*). However, carotenoid content has been related to antioxidant activity in other cyanobacteria genera (*6*), and its presence should be taken into account. Several carotenoids have been described in cyanobacteria, where they have two important roles: they serve as light-harvesting pigments in photosynthesis, and they protect cells against photooxidative damage. Carotenoids also form part of the cyanobacterial cytoplasmic membranes. β-Carotene, *cis*-β-carotene, myxoxanthopyll, zeaxanthin, echinenone, canthaxanthin, nostoxanthin, and chlorophyll *a* have been identified in *Phormidium* species (*27, 28*).

As can be seen in **Figure 1** the main carotenoids content decreased as the extraction temperature increased in hexane extracts, whereas no such differences were recorded for the ethanol extracts; only a slight higher content of lutein/zeaxanthin could be attributed to ethanol extraction at 150 °C. Therefore, TEAC values showed a trend similar to that of the carotenoid composition and it seems plausible to conclude that carotenoids content is, at least, the main factor to explain the antioxidant capacity of ethanol and hexane PLE extracts from *Phormidium* species.

In contrast, antioxidant activity of water extracts could be attributed to the production of Maillard reaction compounds during extraction with subcritical water, because these products have been reported to possess a high antioxidant activity (*29*). This supposition seems to be plausible as more elevated water extraction conditions (200 °C) caused a high concentration of brown highly polar compounds corresponding to the highest antioxidant capacity of all the extracts.

HPLC-DAD Characterization of PLE Extracts. Even though hexane extracts showed the highest antioxidant activity, except for water at 200 °C, they have the worst extraction yield. If TEAC values were expressed relative to cell material, the best results would be obtained with ethanol as extraction solvent. Therefore, ethanol extract at 150 °C was submitted to HPLC-DAD analysis to characterize its pigment composition.

β-Carotene was the main component of the ethanol extract at 150 °C (**Table 2**; **Figure 2**). Other identified carotenoids regarding their normalized area percentage were lutein, violaxanthin, and neoxanthin. Echinenone was tentatively identified as one of the main constituents of *Phormidium* sp. extracts. Other carotenoids with a high contribution to total area could not be identified. Some of these unidentified carotenoids could correspond to nostoxanthin, myxoxanthophyll, and isomers of other carotenoids such as canthaxanthin or β-carotene as they have been reported to be found in *Phormidium* species (*27, 28*). Moreover, as expected, chlorophyll *a* and chlorophyll *a* derivatives were also found in this extract.

Antimicrobial Activity of the PLE Extracts. Four different microbial species, including a Gram-negative bacteria (*E. coli*), a Gram-positive bacteria (*S. aureus*), a yeast (*C. albicans*), and a fungus (*A. niger*), were used to screen the potential antimicrobial activity of the *Phormidium* sp. extracts obtained using pressurized solvent extraction. The antimicrobial activity was quantitatively assessed by the determination of the MIC, MBC, and MFC.

The results obtained in all of the conditions employed in this study are shown in **Table 3**. The most sensitive microorganism for all the extracts tested was the yeast, *C. albicans*; meanwhile,

Table 2. HPLC-DAD Tentative Identification (NI, Not Identified) and Peak Area Contribution (Area Counts and Normalized Area Percent) of Compounds Found in the PLE Ethanol Extract at 150 °C of *Phormidium* Species

			spectroscopic data	*Phormidium* sp. 150 °C ethanol	
peak	t_R	identification	characteristic UV maxima[c] spectra profile	area (area counts)	normalized area %
1	5.52	NI carotenoid	418, 442, 472, 658	588.1	1.3
2	5.99	NI carotenoid	420M, 440, 470, 658	433.5	1.0
3	6.42	violaxanthin[a,b]	418, 442, 472, 658	525.2	1.2
4	6.63	neoxanthin[a,b]	416, 438, 468, 656	524.5	1.2
5	12.66	lutein[b]	424, 448, 476, 660	5171.8	11.4
6	14.77	NI carotenoid	430, 454, 478, 662	4821.4	10.7
7	17.23	NI chlorophyll	420, 456, 654	715	1.6
8	17.83	NI chlorophyll	336, 378, 414, 430, 576, 616, 664	584.1	1.3
9	18.97	X + chlorophyll	418, 476, 510, 612, 658	5390.3	11.9
10	22.33	NI	420, 475Sh, 617, 662	785.8	1.7
11	29.17	echinenone	430Sh, 452, 474Sh	6305.7	13.9
12	29.69	echinenone	452	4725.6	10.5
13	31.78	β-carotene[a,b]	430, 454, 480	11092.4	24.5
14	32.47	NI carotenoid	426, 448, 474, 654	3550.8	7.9

[a] Similar spectra as standard. [b] Retention time equal to standard. [c] Sh, shoulder.

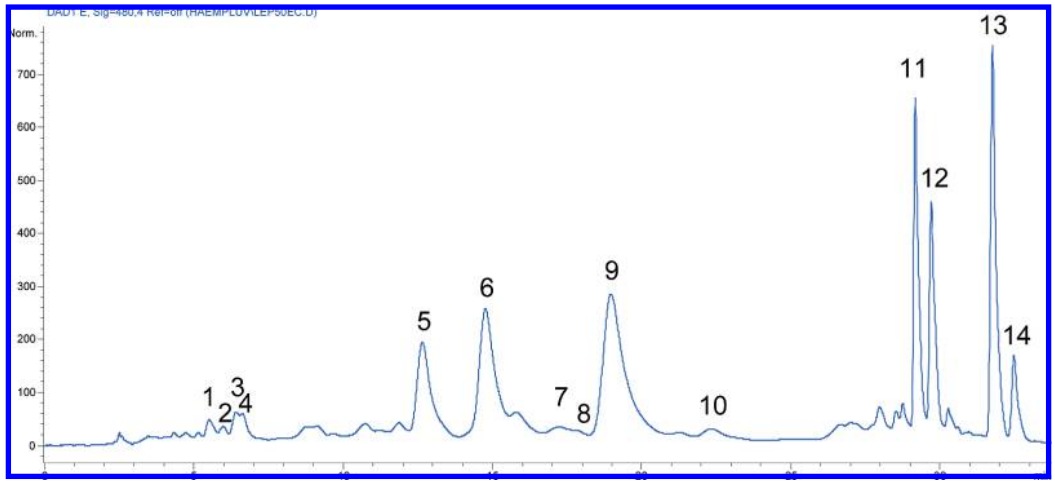

Figure 2. HPLC chromatogram (450 nm) of a pressurized ethanol extract of *Phormidium* sp., obtained at 150 °C and 20 min. Peak assignment is as in **Table 2**. Chromatographic method: C30 analytical column (5 μm, 250 × 4.6 mm i.d.); mobile phase, solvent A (acetone), solvent B (water), gradient (50 min) starting from 16% B (constant for 21 min) to 3% B in 4 min; flow rate, 1 mL/min.

the extracts showed a very small antimicrobial activity against *E. coli*, *S. aureus*, and *A. niger*. In general, no differences in the antimicrobial activity of the extracts were found when the different solvents were used (at the same extraction temperature). These data could indicate that the compounds responsible for the antimicrobial activity of these microalgae presented a diverse chemical nature (polarity). In that way, several authors have attributed the antimicrobial activity of cyanobacteria to different compounds belonging to a diverse range of chemical classes, including indoles, terpenes, acetogenins, phenols, fatty acids, and volatile halogenated hydrocarbons (*1, 2*).

The analysis of the antimicrobial activity of the extracts, as a function of extraction temperature, indicated that an increased in the extraction temperature normally did not produce a significant change in their antimicrobial activity. Nevertheless, with regard to bacteria, extracts obtained at 200 °C seemed to be slightly more active, whereas for ethanol, extracts at 150 °C also presented a higher activity. These data revealed that the extraction temperature was not an important parameter to optimize the extraction of antimicrobial components. Only in the case of bacteria could the use of a high temperature (200 °C) be useful to improve the extraction of compounds with antimicrobial activity.

GC-MS Characterization of PLE Extracts. In an attempt to identify the compounds responsible for the antimicrobial

Table 3. Antimicrobial Activities of Different PLE Extracts from *Phormidium* Species

sample	temperature (°C)	*E. coli* MBC[a]	*S. aureus* MBC	*C. albicans* MFC[b]	*A. niger* MFC
hexane extracts	50	15.0 ± 0.8	16.3 ± 0.6	8.3 ± 0.4	20.1 ± 0.9
	100	15.2 ± 0.5	16.1 ± 0.3	8.0 ± 0.6	20.2 ± 1.0
	150	15.4 ± 0.7	16.0 ± 0.5	8.3 ± 0.4	19.8 ± 0.8
	200	13.1 ± 0.3	11.9 ± 0.4	8.9 ± 0.7	20.1 ± 0.7
ethanol extracts	50	16.4 ± 0.8	17.1 ± 0.7	7.2 ± 0.5	20.3 ± 0.8
	100	16.1 ± 0.5	17.3 ± 0.5	7.2 ± 0.3	20.0 ± 0.7
	150	13.8 ± 0.7	16.2 ± 0.4	7.4 ± 0.2	19.9 ± 0.9
	200	14.0 ± 0.5	16.1 ± 0.2	8.3 ± 0.4	20.0 ± 0.9
water extracts	50	16.5 ± 0.2	17.3 ± 0.5	8.1 ± 0.4	19.9 ± 1.0
	100	16.3 ± 0.8	17.1 ± 0.8	7.8 ± 0.2	20.2 ± 1.1
	150	16.6 ± 0.6	17.4 ± 0.7	7.5 ± 0.3	20.3 ± 0.8
	200	13.7 ± 0.7	15.2 ± 0.6	7.5 ± 0.5	20.0 ± 0.7
reference antibiotic		10.1 ± 0.3	10.4 ± 0.5	100.8 ± 4.9	150.6 ± 6.9

[a] MBC, minimum bactericidal concentration. [b] MFC, minimum fungicidal concentration. MBC and MFC values given as mg/mL for samples and as μg/mL for antibiotic.

Table 4. GC-MS Identification (Not Identified), Peak Area Contribution (Normalized Area Percent), and Retention Indices (RI) of Compounds Found in the PLE Extracts (Ethanol at 50 and 150 °C) from *Phormidium* Species

t_R	identification	RI	Lep50et normalized area (%)	Lep150et normalized area (%)
25.12	β-ionone	1521	0.49	0.30
25.57	phenol, 2,6-bis(1,1-dimethylethyl)-4 methyl	1549	1.50	1.74
25.96	2(4*H*)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl	1574	0.73	0.38
26.79	hexadecane	1620	0.52	0.37
27.93	1-hexadecanol (-2-methyl)	1748	51.03	33.27
29.22	neophytadiene	1895	8.59	8.24
29.27	2-hexadecane, 3,7,11,15-tetramethyl	1901	1.78	1.76
29.42	2-hexadecen-1-ol-3,7,11,15-tetramethyl	1920	1.54	1.39
29.57	neophytadiene deriv	1939	2.57	2.43
30.01	9-hexadecenoic acid	1995	2.86	6.83
30.17	hexadecanoic acid	2019	10.39	16.23
30.22	1,2-benzenedicarboxylic acid, dibutyl ester	2026	2.21	3.37
30.37	hexadecanoic acid, ethyl ester	2049		3.39
31.35	9,12-octadecadienoic acid	2199	14.21	25.06
31.82	phenol, 4,4′-(1-methyl ethylidene)bis-	2279	1.51	1.99
total sum			5400.85	4158.90

activity, a characterization by GC-MS of some of the PLE extracts was performed. Ethanol extracts at 50 and 150 °C were analyzed, because ethanol was a solvent with an intermediate polarity and, against the bacteria *E. coli*, presented the higher influence of the extraction temperature in the antimicrobial activity. Results obtained are shown in **Table 4**, where a tentative identification has been performed on the basis of the comparison of mass spectra and RI.

As can be seen in **Table 4**, 15 compounds have been identified in the two samples, mainly fatty acids, phenols, and compounds such as neophytadiene and phytol. By comparing the composition of the two samples, it can be seen that the amounts of phenols, neophytadiene, phytol, and β-ionone were quite similar, whereas extract obtained at 150 °C contained a higher quantity of fatty acids (linoleic, palmitic, and palmitoleic). The main compound in the sample obtained at 50 °C was 2-methyl-1-hexadecanol (51.03%).

With regard to these data, the presence of neophytadiene, a compound found in an appreciable amount in the extracts (10–11%) and which has been reported to possess antimicrobial activity (*30*) can be noted. However, because the amounts of this substance detected in both samples were similar, the higher antimicrobial activity of the extract at 150 °C could not be attributed to the presence of this compound. Fatty acids have also been reported as potential antimicrobial compounds (*31*). The antimicrobial activity of fatty acids has been usually attributed to long-chain unsaturated fatty acids (C16−C20), including palmitoleic, oleic, linoleic, and linolenic acids, whereas long-chain saturated fatty acids, including palmitic and stearic acids, were less effective (*32*). Therefore, the higher antimicrobial activity found with ethanol extract at 150 °C seemed to be related to the higher quantity of palmitoleic and linoleic acids, compared to the sample extracted at 50 °C.

On the other hand, Volk (*33*) reported that *Phormidium foveolarum* was able to excrete to the culture medium a small quantity of norharmane (an indole alkaloid). This compound has been found to have some pharmacological effects (*34*), including antimicrobial activity against cyanobacteria, Gram-positive bacteria, Gram-negative bacteria, and *C. albicans* (*35*). However, this substance was not found in the two samples analyzed. This fact can be explained because the PLE extractions were carried out from the cyanobacteria cells, excluding their culture medium.

To study the viability of *Phormidium* extracts to be used as functional ingredients, the cytotoxicity of the different extracts was evaluated on preformed monolayers of Vero cells (African green monkey kidney cell line) (ATCC) by the MTT method, as mentioned previously. Data were plotted as dose–response curves, from which the concentration required to reduce 50% the number of viable Vero cells (CC50) after 48 h of incubation with the different extracts was obtained. The CC50 data indicated that *Phormidium* extracts presented a low cytotoxicity (values between 0.99 and 1.63 mg/mL), compared to data obtained for *S. platensis* extracts in the same conditions, which showed much higher cytotoxicity (0.458–0.672 mg/mL).

In conclusion, data presented in this study have pointed out the presence of antioxidant and antimicrobial compounds in *Phormidium* species. Pressurized fluid extraction seems to be a useful technique for the extraction of these functional compounds from *Phormidium* species. Antioxidant activity was mainly associated with carotenoids content, whereas antimicrobial activity seemed to be related to phenolic compounds together with fatty acids content. Therefore, PLE could be used as an environmentally friendly technique for the extraction of functional components from *Phormidium* species.

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