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ANTIMICROBIAL ACTIVITY OF MARINE MICROALGAE ISOLATED FROM MOROCCAN COASTLINES

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ABSTRACT

The present investigation aimed to study antimicrobial activities in marine microalgae, screened from Moroccan coastlines. Ethanolic extracts were prepared from the microalgae and evaluated each against the bacteria: *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, the yeast *Candida albicans* and the fungus *Aspergillus niger*. The highest antibacterial activity was found in the extract of *Tetraselmis* sp. which exhibited an inhibitory effect against the three bacteria with a MIC of 2.6 to 3.0 mg extract per mL culture. Extracts from the other microalgae: *Dunaliella Salina*, *Nannochloropsis gaditana*, *Dunaliella* sp., *Phaeodactylum tricornutum* and *Isochrisis* sp. each showed inhibitory activity against *E. coli* or *P. aeruginosa* with a MIC of 2.6 to 4.3 mg extract per mL. The extract of *N. gaditana* also inhibited *S. aureus* growth. However, the extracts from the microalgae, *Chaetoceros* sp. and *Chlorella* sp. showed no effect under the applied experimental conditions. All the tested extracts inhibited the growth of *C. albicans*; the highest activity was obtained from *N. gaditana* with a MIC of 4.0 mg extract per mL culture. *Aspergillus niger* appeared to be resistant to the effect of the extracts. The observed antimicrobial activities were linked to the contents of the extracts in fatty acids, carotenoids and phenolic compounds. In conclusion, the studied microalgae could be considered as a potential natural source of bioactive compounds with antimicrobial activities.

Keywords: Marine microalgae, antimicrobial activity, phenolic content, carotenoids, fatty acids

INTRODUCTION

There has been increasing demand for new antimicrobial compounds in response to continuous evolution of microbial pathogens in antibiotic-resistance. Marine environment has been considered among the most promising sources of antimicrobial compounds, as numerous sea organisms produce bioactive metabolites in response to environmental stress and develop chemical strategy for defense and survival (**Mhadhebi *et al.*, 2012**). A large number of new active antimicrobial compounds have been isolated from marine sources. But, the majority of these compounds has not been yet characterized (**Sanmukh *et al.*, 2014**).
Marine microalgae constitute attractive sources of novel and active metabolites, comprising proteins, enzymes, pigments and polyunsaturated fatty acids (PUFA) that could be exploited in pharmaceutical, food, feed and cosmetic industries (**Mendes *et al.*, 2003**; **Cardozo *et al.*, 2007**; **Surendhiran *et al.*, 2014**). Compounds with pharmaceutical characteristics, as antioxidative, antiinflammatory, antimicrobial or antitumoral properties, have been identified; some of them have been in the clinical trial state (**Guedes *et al.*, 2011**; **Kwak *et al.*, 2014**). Antimicrobial activities are among the most researched features in natural extracts. They have been attributed to different compounds, including, indoles, terpene derivatives, acetogenins, phenols, fatty acids and hydrocarbons (**Bhakuni and Rawat, 2005**; **Santoyo *et al.*, 2009**).
Selected examples from the studied antimicrobial activities in microalgae are summarized in Table 1. However, to our knowledge, there are no data available on the antimicrobial potential of microalgae, isolated from the Moroccan coastlines. In order to evaluate this potential, nine microalgae, collected from these coastlines and identified, were extracted with ethanol; the collected extracts were examined against microbial targets. The obtained results are described hereafter, and discussed with consideration to the previously determined contents of the microalgal extracts in fatty acids, carotenoids and phenolic compounds (**Maadane *et al.*, 2015**).

Table 1 Extracts and compounds from microalgae with antimicrobial activity

Microalgal species	Active extracts/compounds	Target microorganisms	References
Phaeodactylum tricornutum	Eicosapentaenoic acid	*Listonella anguillarum* *Lactococcus garvieae* *Staphylococcus aureus* *Vibrio* sp.	(**Desbois *et al.*, 2009**) (**Smith *et al.*, 2010**)
Dunaliella salina	Faty acids	*Staphylococcus aureus* *Candida albicans*	(**Herrero *et al.*, 2006**)
Haematococcus pluvialis	Short chain fatty acids (butanoic acid, methyl lactate)	*Escherichia coli* *Staphylococcus aureus Candida albicans*	(**Santoyo *et al.*, 2009**)
Chloroccum humicola *Spiriluna platensis*	Pigments	*E. coli* *Staphylococcus aureus* *Bacillus subtilis* *Streptococcus* sp. *Bacilus* sp. *Pseudomonas* sp.	(**Bhagavathy *et al.*, 2011**) (**Muthulakshmi *et al.*, 2012**)

		Staphylococcus sp. *E. coli*	
Anabena sphaerica *Oscillatori alimentica* *Spirulina platensis*	Polyphenols	*E. coli* *Staphylococcus aureus*	(**Klejdus *et al.*, 2010**) (**Hetta *et al.*, 2014**)
Pithophora oedogonium	Ethanolic extract	*Salmonella* *Staphylococcus* sp.	(**Danyal *et al.*, 2013**)
Tetraselmis suecica	Fatty acids	*Vibrio cholerae, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Salmonella* sp. *Proteus* sp. *Streptococcus pyogens, Staphylococcus aureus, Bacillus megaterium* *Bacillus subtilis*	(**Dooslin Mercy Bai and Krishnakumar, 2013**)

MATERIALS AND METHODS

Microalgae and culture conditions

Nine marine microalgae were selected for this study from the collection of Moroccan Foundation for Advanced Science, Innovation & Research (MAScIR, Rabat). They were identified (**Maadane *et al.*, 2015**) as: *Nannochloropsis gaditana*, *Dunaliella salina*, *Dunaliella* sp., *Phaedactylum tricornutum*, *Isochrysis* sp., *Navicula* sp., *Chaeotoceros* sp., *Chlorella* sp. and *Tetraselmis* sp. Culturing conditions of the microalgae were previously described (**Maadane *et al.*, 2015**). Briefly, batch cultures were realized in 5 L flasks containing sterile natural seawater, enriched with F/2 medium nutrients; the cultures were agitated by air bubbling at 25 °C, under continuous illumination at intensity of 150 $\mu mol.m^{-2}.sec^{-1}$; microalgal biomasses were harvested by centrifugation, freeze-dried and stored at -20° until use.

Extraction of microalgal substances

Crude extracts from the microalgae were prepared by extracting 1 g of dried biomass with 100 ml of ethanol for 3 h at room temperature, in the darkness. The extraction was repeated twice for each alga; collected extracts were combined into one sample. The samples were then filtered and concentrated under reduced pressure in a rotary evaporator. The resulting concentrated extracts were stored at -20°C until use.

Extract antimicrobial activity evaluation

The microalgal extracts were tested against five microorganisms, including two Gram-negative bacteria: *Escherichia coli* (ATCC-8739), *Pseudomonas aeruginosa* (ATCC-9027), one Gram-positive bacterium, *Staphylococcus aureus* (ATCC-6538), the yeast *Candida albicans* (ATCC-10231) and the mold *Aspergillus niger* (ATCC-16404). Spores of the mold were harvested into sterile distilled water from monoconidial cultures, developed on potato dextrose at 24°C during 7 days.

The minimum inhibitory concentrations (MICs) of the extracts were determined against all the tested microorganisms using the broth microdilution method (**Scorzoni *et al.*, 2007**). Microbial samples were prepared by dilution with growth media to obtain inocula at 10^5 colony forming units (CFU) per mL culture. The test cultures were performed in Muller Hinton broth for bacteria, or in Sabouraud dextrose broth for *C. albicans* or *A. Niger*. The culturing media were supplemented with 0.5% Tween-20. Samples of the extracts were prepared by different dilutions in DMSO, and tested at a final concentration ranging from 0.5 to 5 mg extract per mL culture. Microbial growth was allowed in 96-well micro-titration plates by dispensing into each well 180 µL of microbial culture and 20 µL of microalgal extract sample at various concentrations. The final DMSO amount, being less than 4%, was without growth inhibition effect. Sample blanks were prepared for all the extracts by adding 20 µL of each extract sample to 180 µL of Mueller Hinton (or Sabouraud dextrose) broth medium. The plates were incubated at 37°C, 24 h for the bacteria and 48 h for *C. albicans* or *A. niger*. The antibiotics, chloramphenicol and amphotericin B were used as positive controls against the bacteria or *C. albicans* and *A. niger*, respectively. The viability of the examined microorganisms was assessed by measuring absorbance of cultures at 600 nm against the Mueller Hinton or the Sabouraud dextrose broth, using a Multiscan Spectrophotometer (Thermo-Fisher Scientific Inc). Assays were carried out in triplicate, and repeated twice. Microbial growth inhibition was expressed in percentage term according to the equation:

$$\left[1 - \left(\frac{A_{sample} - A_{sample\ blank}}{A_{control}}\right)\right] \times 100$$

$A_{control}$: absorbance of the cullture control; A_{sample}: absorbance of the culture test sample; $A_{sample\ blank}$: absorbance of the extract sample in the nutrient broth.

RESULTS AND DISCUSSION

The antimicrobial activity level in the ethanolic extracts of the examined microalgae varied depending on the targeted microorganism and the microalgal strain (Table 2). The extract of *Tetraselmis* sp. exhibited an antimicrobial activity against both Gram-positive and Gram-negative bacteria with the lowest MIC values, ranging from 2.6 to 3.0 mg per mL culture. Meanwhile, the ethanolic extract from *Dunaliella salina* showed an antibacterial activity against only the Gram-negative bacteria, *E. coli* and *P.* aeruginosa. However, the extracts from *N. gaditana*, *Dunaliella* sp. *P. tricornutum*, and *Isochrysis* sp. showed relatively moderate antibacterial activities at the used extract concentrations. *Chlorella* sp. and *Chaetoceros* sp. did not exhibit any inhibitory activity against the tested bacteria, even at the concentration of 5.0 mg extract per mL.

Table 2 Antimicrobial activities of ethanolic extracts of microalgae, isolated from Moroccan coastlines.
MIC values are presented as means ± SD (n = 6). The antimicrobial activity was determined in a culture assay of 0.2 mL, composed of 0.18 mL medium and 0.02 mL extract sample.

Microalgae	**MIC (mg extract per mL culture)**				
	E.coli	***P. aeruginosa***	***S. aureus***	***C. albicans***	***A. niger***
Tetraselmis sp.	2.6±0.2	2.9±0.9	3±0.1	>5	-
Dunaliella Salina	3.4±0.4	2.6±0.8	-	>5	-
Nannochloropsis gaditana	3.4±0.2	>5	4.5±0.6	4.0±0.6	-
Chlorella sp.	-	-	-	>5	-
Dunaliella sp.	>5	4.3±0.1	>5	>5	-
Navicula sp.	>5	>5	>5	>5	-
Phaeodactylum tricornutum	4.3±0.1	>5	>5	4.7±0.1	-
Chaetoceros sp.	-	-	-	>5	-
Isochrisis sp.	3.5±0.2	>5	-	>5	-

>5: MIC is expected to be higher than 5 mg extract per mL culture. -: absence of antimicrobial effect at the highest examined extract concentration, 5 mg per mL culture.

Only, *N. gaditana* and *P. tricornutum* showed an activity against *C. albicans* (Table 2). Extracts of the other microalgae had weak activity against this yeast, as at the maximal concentration used in the test, being 5.0 mg per mL culture, the growth inhibition was less than 50%. Therefore, the MICs of these extracts against *C. albicans* were expected to be higher than this concentration. Paradoxically, no antifungal activity was observed in all the extracts against *A. niger* at the tested concentrations.

As noticed under the applied culture conditions, the MICs of the positive controls are in mean values: 0.085, 0.145 and 0.571 mg chloramphenicol per mL culture for respectively *S. aureus*, *E. coli* and *P. aeruginosa*. The MIC of amphotericin against *C. albicans* was much higher, being 1.6 mg per mL culture. For the microalgae that exhibited antimicrobial activities, the registered MICs, which were of 2.6 to 4.7 mg extract per mL (Table 2) are by comparison to those of the antibiotics higher. However, they might be pharmaceutically significant, if the crude state of the tested extracts was considered, as these extracts contained different substances, and only some of them had inhibitory effect. The registered data were in fact concordant with literature-published observations, concerned with the antimicrobial activities of microalgal extracts, as those of *Chaetoceros mulleri* (**Mendiola *et al.*, 2007**), *Chlorella vulgaris* (**Plaza *et al.*, 2012)** and *Nostoc* sp. (**Salem *et al.*, 2014**).

The antimicrobial activities described above could be attributed to different compounds, comprising those previously determined (**Maadane *et al.*, 2015**): fatty acids, carotenoids and phenolic compounds. Fatty acids, which constitute major parts of the extracted biomasses, are particularly considered because their antimicrobial effects have been long recognized (e.g., **Galbraith *et al.*, 1971**; **Desbois *et al.*, 2009**; **Cakmak *et al.*, 2014**).

The extract from *Tetraselmis* sp. contains high amounts of oleic acid (*cis*-9-octadecenoic acid) (48.8 % of the ethanol-extracted fatty acids), linoleic acid (*cis, cis*-9,12-octadecadienoic acid) (36.4 %) and palmitic acid (hexadecanoic acid) (18.6%). These fatty acids were determined as major components in the ethanolic-extract or hexanic-extract of *Dunaliella salina* (**Herrero *et al.*, 2006**), and reported to be responsible, in a main part, of the antimicrobial activity, exercised against *E. coli*, *S. aureus*, and *C. albicans*. Besides, the antibacterial activities observed in the algal species, *Nostoc spongiforme*, *Oscillatoria tenius and Chlorococcus* sp. were linked to their contents in fatty acids (**Suresh *et al.*, 2014**). Considering these literature-data, the antibacterial activity of *Tetraselmis* sp. (Table 2) can be linked, for a major part, to its content in palmitic, oleic and linoleic acids.

The analysis of the ethanolic extract from *D. salina* showed that this microalga contains the highest amount of ethanol extractable PUFA, being 76.9 % of which linolenic acid (*all-cis*-9,12,15-octadecatrienoic acid) constitutes a high part (45.3%) (**Maadane *et al.*, 2015**). According to Lee *et al.* (**2002**), the antimicrobial activity of linolenic acid was high against Gram-positive bacteria, but low against Gram-negative bacteria. The ethanolic extract of *D. salina* (Table 2) showed antibacterial activity against the Gram-negative bacteria, *E. coli* and *P. aeruginosa* and no activity against the Gram-positive bacterium *S. aureus*. If this activity was mainly attributed to the linolenic acid which was found to be abundant in the tested extract, then our observations regarding *S. aureus* are in contrast to those by Lee *et al*. (**2002**).

The extract from *N. gaditana* was shown to contain important amounts of palmitoleic acid (*cis*-9-hexadecenoic acid) (28%), palmitic acid (24.1%), oleic acid (15.3%) and eicosapentaenoic acid (14%). Also, *P. tricornutum* extract was shown to be rich in these acids. Their antimicrobial activities against bacteria and *C. albicans* (Table 2) might be due for a great part to these dominant fatty acids, in agreement with published observations (**Surendhiran *et al.*, 2014**), concerned with the antibacterial activity of the C16-C20 fatty acids-rich extract from *N. oculata*. Especially, eicosapentaenoic acid, dominant in extracts of *P. tricornutum* must have considerable antimicrobial effect (**Desbois *et al.*, 2009**).

Astaxanthin, a carotenoid pigment, was demonstrated to have a significant effect against both Gram-negative and Gram-positive bacteria (**Ushakumari and Ramanujam, 2013**). Besides, different extracted carotenoids from microalgae, comprising *D. salina* (**Herrero *et al.*, 2006**) and *Chlorococcum humicola* (**Bhagavathy *et al.*, 2011**) have been reported to possess important antimicrobial activities, effecting Gram-positive and Gram-negative bacteria and *C. albicans*. According to our previous data (**Maadane *et al.*, 2015**), only the ethanolic extracts from *Dunaliella* sp., *P. tricornutum* and *Tetraselmis* sp. contained carotenoids at significant amounts, being 10.8, 6.3, and 4.6 mg per g extract, respectively. Consequently, the observed antimicrobial activities of these extracts could be due not only to their fatty acids, but also to their carotenoids, in concordance with the referenced data. Besides, the ethanol extracts from *N. gaditana*, *Tetraselmis* sp. and *P. tricornutum* contained high content of phenolic compounds (polyphenols), being 32, 25.5 and16.8 mg, expressed in gallic acid equivalent per g extracted biomass, respectively. These extracts exhibited the highest antimicrobial activity (Table 2) which could be attributed, for a part, to their phenolic compounds in agreement with published observations (e.g., **Pane *et al.*, 2015**).

Bioactive compounds released by microalgal cells are either bactericidal or bacteriostatic (**Falaise *et al.*, 2016**). Their action mechanisms are still poorly understood. However, action modes have been suggested for growth inhibition or killing of bacteria by some of the functional molecules (**Shannon and Abu-Ghannam, 2016**). In this, phytochemicals, comprising microalgal substances, may act by inducing cellular membrane perturbations, interference with certain microbial metabolic processes, modulation of signal transduction or gene expression. Free fatty acids could initiate peroxidative processes, and preclude the synthesis of bacterial fatty acids (**Zheng *et al.*, 2005**; **Desbois and Smith, 2010**). Besides, free fatty acids might interact with cellular membranes of microbial cells, causing leakage of molecules from these cells, reduction of their nutrient uptake or inhibition of their respiration (**Suresh *et al.*, 2014**).

In the present study the antimicrobial activities of the studied microalgal extracts were attributed to their contents of fatty acids, carotenoids and polyphenols, as discussed above. These substances probably act together, either in an independent or synergistic manner. Whatever their action mode, the data, described here, demonstrated the presence of pharmaceutically promising antibacterial compounds in the screened microalgae from the Moroccan costlines.

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