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## Screening of antimicrobial activities in red, green and brown macroalgae from Gran Canaria (Canary Islands, Spain)

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**Abstract** Extracts from 44 species of seaweed from Gran Canaria (Canary Islands, Spain) were screened for the production of antibacterial and antifungal compounds against a panel of Gram-negative and Gram-positive bacteria, mycobacteria, yeasts and fungi. A total of 28 species displayed antibacterial activity, of which six also showed antifungal activity. *Asparagopsis taxiformis* and *Cymopolia barbata* were the species with the strongest activities against the broadest spectrum of target microorganisms. All the species with antibacterial activity were active against Gram-positive bacteria, whereas only two species, *A. taxiformis* and *Osmundea hybrida*, were active against mycobacteria. The production of secondary metabolites with antimicrobial activities by the macroalgae was also studied under different conditions, although no common trend for bioactivity was observed.

**Keywords** Antibacterial compounds · Antifungal compounds · Bioactivity · Antimicrobial screening · Seaweeds

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### Introduction

As a consequence of an increasing demand for biodiversity in the screening programs seeking therapeutic drugs from natural products, there is now a greater interest in marine organisms, especially algae. The ability of seaweeds to produce secondary metabolites of potential interest has been extensively documented [8, 21]. There are numerous reports of compounds derived from macroalgae with a broad range of biological activities, such as antibiotics, antivirals, antitumorals and anti-inflammatories [22], as well as neurotoxins [13]. Chemical structure types include sterols [1], isoprenoids and other related molecules. In spite of an extensive literature, studies on biocide production refer normally to a limited number of algal species, with some exceptions [5]. Furthermore, the compounds previously studied were extracted directly from field-collected samples of seaweeds, so there are no reported data concerning the ability of these organisms to produce different compounds under different conditions.

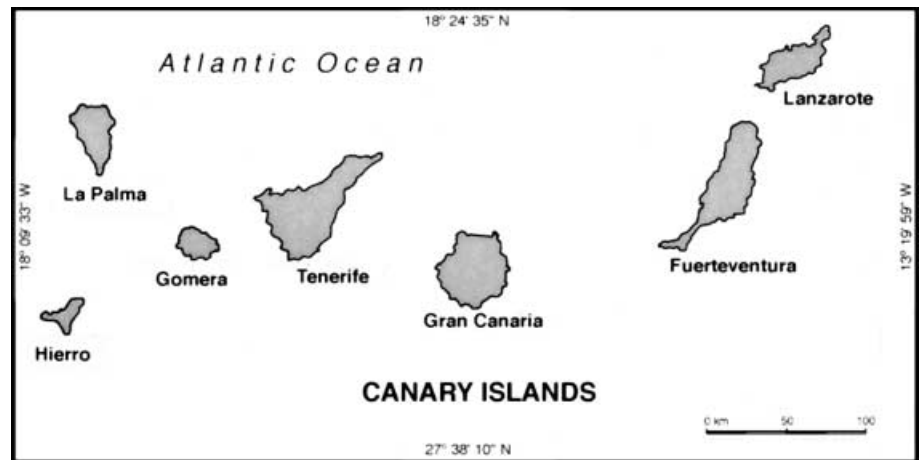
This study assesses the production of secondary metabolites with antimicrobial activity by 44 marine algal species from Gran Canaria island. We also explore the possibility of optimizing the production of biologically active compounds by culturing the seaweeds in bioreactors under different conditions. The cultivation of microorganisms under different conditions has a direct impact on the number and amount of secondary metabolites produced [10]. We wanted to test whether this approach could be also applied to seaweeds, to obtain bioactive metabolites with pharmacological properties.

### Materials and methods

Algal materials and culture conditions

Seaweeds were collected from the north, east and south coasts of the island of Gran Canaria (Canary Islands, Spain; Fig. 1). The

**Fig. 1** Map of the Canary Islands (Spain). Seaweeds were collected from the north, east and south coasts of Gran Canaria



check-lists provided by Alfonso-Carrillo and Sanson [2, 3] and Gil-Rodríguez and Alfonso-Carrillo [12] were used for the classification of the species. Algae were cleaned of epiphytes; and necrotic parts were removed before cultivation. Culture experiments were performed in 80-l Plexiglas cylinders under greenhouse conditions. Seaweeds were grown following the criteria described by Lignell and Peders [14]. Together with field-collected samples (control), two additional experimental growth conditions in the laboratory were established: (1) low stress, where seaweeds cultivated at a density of 2 g/l were exposed to a continuous flow (12 exchanges/day) of natural seawater supplemented with nitrogen (50  $\mu\text{M}$   $\text{NH}_4^+$ ) and (2) high stress, where seaweeds were cultivated at a density of 5 g/l without seawater exchange or nutrients. After cultivation, seaweeds were collected, centrifuged at 1,400 g for 2 min to eliminate superficial water and then frozen at  $-70^\circ\text{C}$ . The whole of the thallus was used for extraction.

#### Methanol extraction

Frozen algal samples were lyophilized and 0.2 g of each were weighed. Then 2 ml of 100% methanol were added, the suspension was shaken for 15 min and then centrifuged at 1,500 g for 15 min. Methanol extracts (prepared in duplicate) were dried under a flow of nitrogen and dissolved in 0.5 ml of 50% methanol, to reduce the toxic effect of the solvent on the test strains.

#### Evaluation of antimicrobial activity

In vitro antimicrobial susceptibility tests were performed using a panel which included both clinical pathogens and laboratory control strains, all of them belonging to the Merck Culture Collection: three Gram-positive bacteria (*Bacillus subtilis* MB964, *Enterococcus faecium* MB5571) and *Staphylococcus aureus* MB5393, two Gram-negative bacteria (*Pseudomonas aeruginosa* MB979 and *Serratia marcescens* MB252), one mycobacterium (*Mycobacterium smegmatis* MB2233), two yeasts (*Candida albicans* MY1055 and *Saccharomyces cerevisiae* W303) and one filamentous fungus (*Aspergillus fumigatus* MF5668). All bacteria used for the tests, except for *B. subtilis*, were resistant to at least one known antimicrobial agent: *Staphylococcus aureus* was methicillin-resistant, *E. faecium* was resistant to vancomycin and  $\beta$ -lactam antibiotics, *M. smegmatis* was resistant to penicillin, aminoglycosides and macrolides; and the two Gram-negative bacterial strains were resistant to penicillin, cephalosporins and macrolides (and, in the case of *P. aeruginosa*, also to imipenem) [4, 7, 25, 28]. *A. fumigatus* MF5668 and *C. albicans* MY1055 are major causes of systemic fungal infections, particularly in immunodepressed patients, including those with acquired immunodeficiency syndrome [27].

The inoculum and assay plates for bacteria, yeast and filamentous fungal strains were prepared as described by Suay [24]. In

all cases, 100-ml aliquots of the seeded agar media were poured into Nunc square plates (24×24 cm).

Methanol extracts (25  $\mu\text{l}$ ) were applied onto the surface of the assay plates seeded with the target microorganisms, which were incubated overnight at  $28^\circ\text{C}$  (yeasts) or  $37^\circ\text{C}$  (bacteria). Inhibition zones around the application points were measured after 24 h. The tests were performed twice, using the two methanol extracts prepared from each sample. The results were in general very similar (differences of less than 3 mm of inhibition zone). An average of the two values was calculated and coded as indicated in the tables. Antibiotics with either positive or negative responses were used as internal controls for the plates (e.g. amphotericin B, gentamycin, hygromycin B, oxytetracyclin, penicillin G and tunicamycin). Methanol (50%) without algal extract was also used as a negative control and, in this case, no antimicrobial activity was observed.

#### Data analyses

Statistical analyses of the distribution of the antimicrobial activities were carried out using the  $\chi^2$  test. The data were contrasted with the expected distribution under the null hypothesis, i.e. the same proportion of active species in each taxon.

**Table 1** Distribution of antimicrobial activities among Divisions (Div.) and Orders of seaweeds

Order	Number of species tested	Active species	Species with antibacterial activity	Species with antifungal activity
Div. Chlorophyta	11	8	8	3
Caulerpales	3	3	3	1
Codiales	4	2	2	0
Dasycladales	1	1	1	1
Ulvales	3	2	2	1
Div. Phaeophyta	17	9	9	1
Dictyotales	8	7	7	1
Ectocarpales	6	1	1	0
Sphacelariales	3	1	1	0
Div. Rhodophyta	16	11	11	2
Ceramiales	3	3	3	1
Corallinales	4	2	2	0
Cryptonemiales	1	0	0	0
Gelidiales	2	1	1	0
Gigartinales	1	1	1	0
Nemaliales	5	4	4	1
Total isolates	44	28	28	6



Besides their antibacterial activity, six species also caused inhibition of the growth of yeasts or filamentous fungi, but none of the species tested showed specific antifungal activity alone. Activity against Gram-negative bacteria was less common than against Gram-positive (one active species, *Asparagopsis taxiformis*, and 28 active species, respectively). However, among the Gram-positive bacteria, not all the target strains tested were equally susceptible to the antimicrobial metabolites produced. The two most susceptible organisms were *B. subtilis* MB964 and *S. aureus* MB5393, which were inhibited by extracts of 22 species and 20 species, respectively. In contrast, only two species (*A. taxiformis* and *Cymopolia barbata*) showed activity against *E. faecium*; and only two species (*A. taxiformis* and *Osmundea hybrida*) were active against *M. smegmatis*. Regarding antifungal activity, six of the species tested (*A. taxiformis*, *C. barbata*, *Caulerpa prolifera*, *Dictyota* sp. 1, *Enteromorpha muscoides* and *O. hybrida*.) presented activity against the filamentous fungus, *Aspergillus fumigatus*, and/or the yeasts *Candida albicans* and *S. cerevisiae*. *Asparagopsis taxiformis* and *Cymopolia barbata* had the strongest antifungal activity. No clear trend in the production of antimicrobial activities by congeneric species

was observed, probably due to the few specimens studied. For instance, different results were obtained among species of *Codium*, *Enteromorpha*, *Halopteris*, *Liagora* and *Sargassum*.

To analyze the effect of different growth conditions on the production of antimicrobial activities by algae, 32 of the species used in this study were cultured in bioreactors (see Materials and methods). Out of 32 species screened, 17 showed activity in the three growth states considered. Six species showed remarkable differences in their activities among the different physiological states tested (Table 3). As for the 17 species active in all states tested, only in the cases of *Dictyota* sp. 1 and *Cymopolia barbata* was there a clear decrease in the inhibitory effect from the natural to the high-stress state, at least against some of the target strains. No activities were found against *M. smegmatis* MB2233, *P. aeruginosa* MB979 and *Serratia marcescens* MB252. For the rest of the species, we observed small differences in intensity between the activities obtained from the field-collected samples and those from cultured seaweeds (data not shown), although it is difficult to conclude whether those differences are significant.

**Table 3** Antimicrobial activities (micro-organisms and classification as in Table 2) in species of seaweed grown under different conditions. *Field* Plant collected from its natural medium and desiccated, *low stress* cultured under low stress conditions for 10 days, *high stress* cultured under high stress conditions for 10 days

Species tested	Target micro-organism
<i>Amphiroa rigida</i>	
Field	Sta (+)
Low stress, high stress	None
<i>Caulerpa webbiana</i>	
Field, low stress	None
High stress	Sta (+)
<i>Codium taylorii</i>	
Field, high stress	None
Low stress	Sta (++)
<i>Cymopolia barbata</i>	
Field	Ent (+++), Sta (+++), Bac (+++), Can (++), Sac (+++), Asp (++)
Low stress, high stress	Ent (+++), Sta (+++), Bac (+++), Can (++), Sac (+++), Asp (+)
<i>Dictyota</i> sp. 1	
Field	Sta (+++), Bac (+++), Can (+), Asp (+)
Low stress, high stress	Sta (++), Bac (++)
<i>Dictyota ciliolata</i>	
Field	None
Low stress	Bac (++)
High stress	Sta (+), Bac (+++)
<i>Gelidium arbuscula</i>	
Field	Bac (+)
Low stress, high stress	None
<i>Sargassum furcatum</i>	
Field	Bac (+++)
Low stress	None
High stress	Bac (+)
<i>Solieria filiformis</i>	
Field	Sta (+++), Bac (+++)
Low stress	Sta (+++)
High stress	Bac (+++)

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## Discussion

The main objective of this work was to evaluate and compare the ability of different macroalgal species from Gran Canaria island to produce bioactive compounds of potential therapeutic interest. The production of antimicrobial activities was considered to be an indicator of the capability of the seaweeds to synthesize bioactive secondary metabolites. The percentages of activities observed for the three algal groups studied (Chlorophyta, Rhodophyta, Phaeophyta) were substantially higher than the data reported in a previous study on macroalgae from Gran Canaria island [6], but were close to those described in another study with seaweeds from the Caribbean Sea [5].

The higher frequency of activity against Gram-positive bacteria has also been observed in most of the surveys of antimicrobial activities from seaweeds reported in the literature [5, 17]. Only one species, *Asparagopsis taxiformis*, showed activity against the whole panel of nine target microorganisms. Although the broad-spectrum antimicrobial activity of *A. taxiformis* against *B. subtilis*, *C. albicans*, *P. aeruginosa* and *S. aureus* has been already described [5], this is the first report of its activity against the other target strains utilized in this study.

The greatest range of activity observed within Orders was found in the Dictyotales, Nemaliales, Ceramiales and Caulerpales. The noteworthy capability of Dictyotales and Caulerpales to produce antimicrobial activities has already been reported [5, 11, 16], but not that of Nemaliales. When compared with other similar studies in the literature, it should be noted that the number of species tested in this work is far higher, with few exceptions [5, 17]. The overlap of species between our study and others covering other geographical locations [5, 15, 23, 26] is very low (less than seven species in common). This is likely due to the limited geographical distribution of most of the species tested in this work. Other reports of the antimicrobial activities of macroalgae from Gran Canaria have been published [6, 9], but the overlapping of species is also quite low (less than 13 species), and they were tested against a panel of fewer microbial strains.

Previous screening studies of antimicrobial activities from macroalgae have detected activities in some of the species tested in this work. Furthermore, some of the species tested in our study have been described as having activities that were not detected in our screening. Some of the species active in our study (*Dictyopteris membranacea*, *Halopitys incurvus*, *Padina pavonica* and *Solieria filiformis*) were not detected as such in previous studies [5, 6, 16]. Species not active in our study nor in other studies [6, 16] are *Cystoseira compressa*, *C. humilis*, *Grateloupia doryphora* and *Jania rubens*. These remarkable differences between our results and those from other studies (as well as between those other studies) may be due to several factors. These could be an infraspecific variability in the production of secondary

metabolites [9, 26], occasionally related to seasonal variations [23]; and there could also be differences in the capability of the extraction protocols to recover the active metabolites and differences in the assay methods, that would result in different susceptibilities of the target strains.

As for the effectiveness of the extraction methods, some studies showed that methanol extraction yielded higher antimicrobial activity than *n*-hexane and ethyl acetate [9], whereas in others, chloroform was better than methanol and benzene [20]. It is clear that the use of organic solvents always provides a higher efficiency in extracting antimicrobial activities, compared with water extraction [19]. We selected methanol extraction for this study, based on our previous experience in the screening of antimicrobial compounds. Given the ability of macroalgae to produce bioactive compounds, it was interesting to test whether this production could be optimized or modulated by culture techniques. There is no reported evidence concerning their capability to produce different compounds depending on their physiological state, although the production of bioactive oxylipins by cell cultures of *Laminaria saccharina* in bioreactors has been described [18]. According to our results, there are remarkable differences in activity among some of the species tested, in their three physiological states. However, there is no clear common trend in their activities related to the physiological state of the algae.

In summary, our results indicate that these species of seaweed collected from Gran Canaria present a significant capacity to show a variety of antimicrobial activities, which makes them interesting for programs screening natural products. This ability is not restricted to one Order or Division within the macroalgae: all of them offer opportunities for producing new types of bioactive compounds. In addition, their capacities could be altered depending on the physiological state of the algae, although it is not clear how this technique should be used to optimize production in general.

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