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Antibacterial activities of marine algae from the Atlantic Coast of Morocco

Activités antibactériennes d'algues marines de la côte atlantique marocaine

Samira Etahiri*, Valérie Bultel-Poncé***[1], Abd Elkbir Elkouri*, Omar Assobhei**, Driss Zaoui*, Michèle Guyot***

* Laboratoire de biochimie marine ; Faculté des sciences, Université Chouaib Doukkali, BP 20, El Jadida, Maroc

** Laboratoire de microbiologie appliquée et biotechnologie, Faculté des sciences, Université Chouaib Doukkali, BP 20 El Jadida, Maroc

*** Laboratoire de chimie des substances naturelles, ESA 8041 CNRS, Muséum national d'histoire naturelle, 63, rue Buffon, 75005 Paris, France

(1) Corresponding author: bultel@mnhn.fr

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ABSTRACT

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Aqueous and organic extracts of twenty-five marine algae collected along the Atlantic Coast of Morocco (Rhodophyceae, Phaeophyceae, Chlorophyceae), were tested for antibacterial activity against different Gram-positive and Gram-negative bacteria. This study shows that most of the algal extracts were significantly active. The highest rates of antibacterial activity were found in Phaeophyceae, followed by the Chlorophyceae and then Rhodophyceae. In this preliminary screening, Gram-positive bacteria were more sensitive than Gram-negative bacteria, and Staphylococcus aureus *was the most susceptible microorganism. Among the extracts, methanolic extracts showed the greatest antibacterial activity (against one or more microorganisms). Among the aqueous extracts tested only those from* Corallina elongata, Sphaerococcus coronopifolius *and* Gracilaria multipartita *presented marked antibacterial activity. Seasonal variation in activity was investigated for the three classes of algae, the greatest activity was observed for samples collected in the Spring.*

RÉSUMÉ

Etahiri S., V. Bultel-Poncé, A.E. Elkouri, O. Assobhei, D. Zaoui, M. Guyot, 2003 - [Activités antibactériennes d'algues marines de la côte atlantique marocaine]. Mar. Life **13** *(1-2) : 3-10.*

Les extraits aqueux et organiques de vingt-cinq algues récoltées sur la côte atlantique marocaine (Rhodophyceae, Phaeophyceae, Chlorophyceae), ont été testés sur différentes souches bactériennes Gram+ et Gram-. La majorité des extraits réalisés à partir des Phaeophyceae a montré une activité antibactérienne significative viennent ensuite les Chlorophyceae et les Rhodophyceae. Dans cette étude, les bactéries Gram+ se sont révélées plus sensibles, en particulier Staphylococcus aureus. *Les extraits méthanoliques se sont révélés les plus actifs, inhibant la croissance d'un ou plusieurs micro-organismes. Seuls les extraits aqueux de* Corallina elongata, Sphaerococcus coronopifolius *et* Gracilaria multipartita *ont montré une activité antibactérienne significative. La variation saisonnière de l'activité a été évaluée pour les trois classes d'algues, les activités les plus significatives étant observées pour les échantillons récoltés au printemps.*

INTRODUCTION

Marine organisms are an important source of biologically active compounds (Caccamese, Toscano, 1982; Glombitza, Koch, 1989; Faulkner, 1999). Macroalgae were extensively studied in the 1970s, but interest in these chemical studies has declined today in favour of the invertebrates. Macroalgae represent a large reservoir of molecules which can be directly exploited (Faulkner, 1993) or produced by biotechnology (Renn, 1993). Antibacterial and antifungal activity has been observed in extracts of a large number of marine algae collected in various parts of the world, from the coast of Brazil, Campos-Takaki *et al.* (1988); from the French Mediterranean coast, Pesando, Caram (1984); from Italy, Caccamese, Azzolina (1979); Cacamese *et al.* (1980, 1981); from India, Rao, Parekh, (1981); Padmakumar, (1994); Crasta *et al.* (1997); from Spain, Ballesteros *et al.* (1992) and from South Africa, Vlachos *et al.* (1997). Bioactive substances previously isolated from algae have been reviewed by Caccamese, Toscano (1982); Glombitza, Koch (1989) and Faulkner (1999). Nevertheless, little is known about algae from the Atlantic coast of Morocco: there have been two studies on the genus *Cystoseira*, Valls *et al.* (1992, 1993) and we have just published an article on the bioactive compounds isolated from *Sphaerococcus coronopifolius*, Etahiri *et al.* (2001).

In this study we have tested aqueous and organic algal extracts of twenty-five marine algae, Rhodophyceae, Phaeophyceae, Chlorophyceae (Benhissoune *et al.*, 2001), collected along the Atlantic Coast of Morocco, for antibacterial activity. In addition, we have compared antibacterial activity of samples collected in two different seasons. Most of the species collected have not been studied before, only seven of them were mentioned in earlier works. The goal of our program was to evaluate the pharmacological potential of algal extracts from the Atlantic Coast of Morocco, in order to isolate and characterise bioactive compounds.

MATERIAL AND METHODS

Sample collection

Algae were collected from the El Jadida coast (33°-33°16'09'' N; 8°30'-8°45' W) in Morocco during two different periods (March-May 1997 and December 1997-January 1998) to evaluate seasonal effects on antibacterial activity. Voucher specimens were preserved in 5% formaldehyde until identification by Dr de Revier, and deposition at PC - Laboratoire de cryptogamie, 12, rue Buffon, 75005 Paris, France.

Preparation of extracts

Samples were washed with distilled water, cleaned of extraneous matter, then air dried at room temperature and in darkness for three days. Each sample was ground and divided into five parts, which were separately extracted, according to Caccamese, Azzolina (1979), by shaking with methanol, acetone, chloroform and hexane (1 g of alga in 5 mL of solvent) for a night, and then centrifuged. The supernatants were concentrated under reduced pressure and aliquots prepared to test antibacterial activity. Extracts were stored at 4°C.

Powdered alga was extracted overnight with distilled water in the proportion of 1 g of alga per 10 mL at room temperature. After centrifugation, aqueous extract was stored at 4°C before screening.

Screening for antibacterial activity

Extracts were tested against Gram-positive and Gram-negative bacterial strains obtained from the Institut Pasteur Collection (IPC, Paris, France) and American Type Culture Collection (ATCC, Rockville, MD, USA): *Bacillus thuringiensis* (Bt) ATCC 10792; *Bacillus cereus* (Bc) CIP 783; *Bacillus subtilis* (Bs1) ATCC 9372; *Bacillus subtilis* (Bs2) ATCC 6633; *Staphylococcus aureus* (Sa1) ATCC 9144; *Staphylococcus aureus ssp aureus* (Sa2) ATCC 6538; *Clostridium sporogenes* (Cs) CIP 7939; *Mycobacterium smegmatis* (Ms) CIP 7326 as Gram-positive species, and *Escherichia coli* (Ec) ATCC 10536 as Gram-negative species.

Antibacterial assays were carried out using the agar disk-diffusion assay as described by Bauer *et al.* (1966). Three colonies of the organism to be tested were picked with a wire loop from the original culture plate and were introduced into a test tube containing 5 mL of broth. An overnight culture yielded a suspension of 10^6 bacteria.mL^{-1} (evaluated by the absorbance value of 0.5 at the wavelength of 620 nm). This solution was diluted 100-fold and the bacteria density was then adjusted to 0.2×10^4 cells.mL^{-1} with sterile water to inoculate Petri dishes containing culture media (12 mL of Mueller-Hinton agar, 3 mm of thickness). Plates were dried for about 30 min before inoculation and were used within four days of preparation.

Organic extracts were tested using paper disks (6 mm diameter) impregnated with the solution (500 µg/disk), while aqueous extracts were tested according to the "well assay" (Chabbert, 1963) using a solution of extracts (concentration of 500 µg.50 µL^{-1}) in each well (well volume=100 µL). After the temperature was equalized at 4°C, the microorganisms were incubated overnight at 37°C. Diameters of inhibitory zones were then measured. Further investigations were carried out with standard antibiotics, chloramphenicol, tetracycline and streptomycin. Control disks were prepared with each solvent. Tests were performed in duplicate.

RESULTS AND DISCUSSION

Table I lists results obtained for each extract against one or more microorganisms. 52% of the marine algae tested showed significant antibacterial

Table I - Screening of algae extracts for their antibacterial activity. [a]Bc: *Bacillus cereus*; Bs1: *Bacillus subtilis* 1; Bs2: *Bacillus subtilis* 2; Bt: *Bacillus thuringiensis*; Cs: *Clostridium sporogenes*; Sa1: *Staphylococcus aureus* 1; Sa2: *Staphylococcus aureus* ssp *aureus*; Ec: *Escherichia coli*; Ms: *Mycobacterium smegmatis*. [b]Diameter of inhibition zone: + <10 mm; ++ 10-15 mm; +++ >15 mm; T: trace; "-": no activity; ND: not determined. / *Criblage de l'activité antibactérienne des extraits d'algues.* [a]*Bc :* Bacillus cereus ; *Bs1* : Bacillus subtilis *1 ; Bs2 :* Bacillus subtilis *2 ; Bt :* Bacillus thuringiensis *; Cs :* Clostridium sporogenes *; Sa1 :* Staphylococcus aureus *1 ; Sa2 :* Staphylococcus aureus *ssp aureus ; Ec :* Escherichia coli *; Ms :* Mycobacterium smegmatis. [b]*Diamètre de la zone d'inhibition : + <10 mm ; ++ 10-15 mm ; +++ >15 mm ; T : trace ; "-" : pas d'activité ; ND : non déterminé.*

Algae	[a]Bacteria	[b]Inhibitory activity of extracts in the following solvents				
		Methanol	Acetone	Chloroform	Hexane	Water
Chlorophyceae						
Enteromorpha clathrata (Roth) Greville	Cs	++	+	-	-	-
	Sa2	++	+++	-	-	-
	Ms	++++	-	-	-	-
Enteromorpha intestinalis (Linnaeus) Nees	Sa2	++	++	T	-	-
Enteromorpha muscoides (Clemente y Rubio) Cremades	Bc	+	T	-	-	-
	Sa2	++	++	T	-	-
Rhizoclonium riparium (Roth) Harvey	Sa2	+++	+	++	-	-
Ulva rigida C. Agardh	Bc	++	T	-	-	-
	Sa2	++	-	T	-	-
	Ms	++	T	-	-	-
	Ec	-	-	T	-	-
Phaeophyceae						
Bifurcaria bifurcata Ross	Bc	+	++	-	+	-
	Bt	++	+++	-	+	-
	Bs1	+	++	-	+	-
	Cs	+++	++	-	-	-
	Sa2	+++	+	T	-	-
Colpomenia sinuosa (Mert) Derbes and Solier	Sa1	++	++	+	-	-
Cystoseira tamariscifolia (Hudson) Papenfuss	Cs	++	+	-	+	-
	Sa2	++	+++	++	-	-
Cystoseira humilis Küntzing	Bc	+++	++	-	+	-
	Bt	+++	+++	-	+	-
	Bs1	+++	++	-	+	-
	Bs2	+++	++	-	-	-
	Cs	++	++	-	-	-
	Sa1	+++	+++	+	-	-
	Sa2	+++	++	-	-	-
	Ms	+++	++	-	-	-
Fucus spiralis Linnaeus	Bc	+	+	-	-	-
	Bt	-	++	-	-	-
	Cs	+++	++	-	-	-
	Sa2	+++	-	-	-	-
	Ec	-	++	-	-	-
Laminaria ochroleuca de la Pylaie	Sa2	++	+++	+	-	-
Sargassum vulgare C. Agardh	Bc	+	+	-	-	-
	Cs	+	++	-	-	-
	Sa2	+++	+++	++	-	-
Rhodophyceae						
Asparagopsis armata Harvey	Bc	++	+	-	-	-
	Cs	++	+	-	-	-
	Sa2	-	+++	++	-	-
	Ec	-	-	++	-	-
Bonetia secundiflora (J. Agardh) Thuret	Sa2	++	++	++	-	-
Caulacanthus ustulatus (Mert) Kützing	Sa2	++	-	+	-	-
Corallina elongata Ellis and Solander	Bc	+	+	-	-	-
	Bs1	+	+	-	-	-
	Bs2	-	+	-	-	-
	Bt	+	+	-	-	-
	Cs	+	+	-	-	-
	Sa2	++	+++	++	++	++
	Ec	-	-	++	-	-
Gelidium latifolium (Greville) Bornet and Thuret	Bs1	T	T	-	++	-
	Sa2	++	++	-	-	-
Gigartina acicularis (Roth) Lamouroux	Sa2	T	T	++	-	-
Gigartina teedi (Roth) Lamouroux	Sa2	++	T	++	-	-
Gracilaria multipartita (Clemente y Rubio) Harvey	Cs	+	T	-	T	+
	Sa2	++	++	-	-	+
Gracilaria verrucosa (Hudson) Papenfuss	Bc	+	T	ND	-	T
	Sa2	+++	++	T	-	-
Halopitys incurvus (Hudson) Batters	Bc	++	+	-	-	-
	Cs	++	++	+	-	-
	Sa2	+++	-	-	-	-
	Ec	-	-	T	-	-
Hypnea musciformis (Wulfen) Lamouroux	Bs2	T	T	-	-	-
	Sa2	++	T	-	-	-
Plocamium cartilagineum (Linnaeus) Dixon	Bs2	T	+	-	-	-
	Sa2	++	+	-	-	-
Sphaerococcus coronopifolius Stackhouse	Sa2	++	+++	++	++	+
	Ec	-	-	T	-	-

Table II - Summary of activity of extracts from algae towards different strains. [a]Bc: *Bacillus cereus*; Bs1: *Bacillus subtilis* 1; Bs2: *Bacillus subtilis* 2; Bt: *Bacillus thuringiensis*; Cs: *Clostridium sporogenes*; Ms: *Mycobacterium smegmatis*; Sa1: *Staphylococcus aureus* 1; Sa2: *Staphylococcus aureus* ssp *aureus*; Ec: *Escherichia coli.* / *Résumé de l'activité des extraits d'algues en fonction des souches cibles.* [a]*Bc :* Bacillus cereus *; Bs1 :* Bacillus subtilis *1 ; Bs2 :* Bacillus subtilis *2 ; Bt :* Bacillus thuringiensis *; Cs :* Clostridium sporogenes *; Ms :* Mycobacterium smegmatis *; Sa1 :* Staphylococcus aureus *1 ; Sa2 :* Staphylococcus aureus *ssp* aureus *; Ec :* Escherichia coli.

Solvents used for extraction	[a]Bacteria									Number of positive assays
	Bc	Bs1	Bs2	Bt	Cs	Ms	Sa1	Sa2	Ec	
Methanol	10	2	1	3	4	3	2	12	-	37
Acetone	7	3	3	4	3	1	1	8	-	30
Chloroform	-	-	-	-	1	-	1	7	2	11
Hexane	1	3	-	2	-	-	-	2	-	8
Aqueous	-	-	-	-	1	-	1	-	-	2
Number of active extracts	18	8	4	9	9	4	5	29	2	

activities, with inhibition diameters greater than 10 mm. The highest number of active species was found in the class of Phaeophyceae: 6 of 7 algae tested were found to be active (85%), followed by Chlorophyceae (40%) and Rhodophyceae (38%). These results are in agreement with those reported by many workers, Caccamese *et al.* (1980, 1981); Pesando, Caram (1984); Vlachos *et al.* (1997), but are in contrast with those of other authors, Rao, Parekh (1981); Mahasneh *et al.* (1995); Padmakumar, Ayyakkannu (1997), who reported highest antibacterial activity rates for Rhodophyceae.

Significant activity has been shown in each class, with inhibition diameters ranging from 10 to 15 mm. Most of the algae tested were ineffective against the Gram-negative strain: only *Fucus spiralis* (Phaeophyceae), *Ulva rigida* (Chlorophyceae), *Sphaerococcus coronopifolius, Corallina elongata, Halopitys incurvus* and *Asparagopsis armata* (Rhodophyceae) presented a low rate of activity against *E. coli.* Caccamese *et al.* (1985); Vidyavathi, Sridhar (1991) and Febles *et al.* (1995) reported a higher susceptibility of the strain *B. subtilis.*

The antibacterial activity is not uniformly distributed in the various extracts (table II): methanolic extracts exhibited greater inhibition against Gram-positive bacteria. In the class of Phaeophyceae, methanolic extracts of *Bifurcaria bifurcata, Cystoseira humilis, Sargassum vulgare, Fucus spiralis* showed the best antibacterial activity against different Gram-positive bacteria especially *S. aureus* and *C. sporogenes* (inhibition diameter larger than 15 mm). In that class, activities were nearly the same for acetonic extracts of *Sargassum vulgare, Laminaria ochroleuca, Bifurcaria bifurcata, Cystoseira humilis* and *Cystoseira tamariscifolia.*

The class of Rhodophyceae was the most represented group, with 52% of algae tested. Methanolic extracts of *Halopitys incurvus* and *Gracilaria verrucosa* showed significant activity against *S. aureus.* Similar activitiy rates were observed for acetonic extracts of *Asparagopsis armata, Corallina elongata* and *Sphaerococcus coronopifolius.* For example antibacterial activity of *Sphaerococcus coronopifolius* was higher than that previously reported by Ballesteros *et al.* (1992): we observed significant activity, with inhibition diameters between 10 to 15 mm, against *B. cereus, S. aureus* and *M. smegmatis.*

In the class of Chlorophyceae, the methanolic extract of *Rhizoclonium riparium* and the acetonic extract of *Enteromorpha clathrata* showed a broad spectrum of activity. Significant antibacterial activity against the mycobacterium *M. smegmatis* was observed with the methanolic extracts of *Enteromorpha clathrata, Ulva rigida* and *Cystoseira humilis. Enteromorpha intestinalis* reported inactive against *S. aureus* by Hornsey, Hide (1974) and Henriquez *et al.* (1979), was found to inhibit the growth of S. aureus in the present study, in agreement with data reported by Padmakumar, Ayyakkannu (1997).

This study demonstrated that algae extracted by solvents such as methanol and acetone showed antibacterial activity, and that greatest inhibition diameters were obtained with methanolic extracts. These results are in agreement with the observations of Febles *et al.* (1995), who reported that extracts prepared with methanol showed the best activity, and those of Delaraisassi *et al.* (1996), who reported that acetone was the best solvent. However they are in contrast with those of Sastry *et al.* (1994), who mentioned chloroform as the most suitable solvent for extracting antibacterial substances from algae. The present investigation revealed that all the Gram-positive organisms tested were sensitive to the methanolic extracts, *S. aureus* appearing more sensitive to the inhibitory effect of the extracts than the other microorganisms. Similar results were obtained by Allen and Dawson (1960) and Rao and Parekh (1981).

Table III - Seasonal variation in the antibacterial activity of marine algae against *S. aureus* 1. [a]A: acetonic extracts; Ch: chloroformic extracts; H: hexanic extracts; M: methanolic extracts; Aq: aqueous extracts. [b]Diameter of inhibition zone: +<10 mm; ++ 10-15 mm; +++ > 15 mm; T: trace; "-": no activity; ND: not determined. / *Variation saisonnière de l'activité antibactérienne des extraits d'algues sur la souche* S. aureus *1. [a]A : extraits acétoniques ; Ch : extraits chloroformiques ; H : extraits hexaniques ; M : extraits methanoliques ; Aq : extraits aqueux. [b]Diamètre des zones d'inhibition : + <10 mm ; ++ 10-15 mm ; +++ > 15 mm ; T = trace ; "-" = pas d'activité ; ND = non determiné.*

Algae	[a]Extract	[b]Antibacterial activities of algae collected in	
		March-May	December-January
Chlorophyceae			
Enteromorpha intestinalis	M	++	+
	A	++	-
	Ch	T	-
Enteromorpha muscoides	M	++	+
	A	++	-
Ulva rigida	M	++	T
Phaeophycaea			
Bifurcaria bifurcata	M	+++	++
	A	++++	+
	Ch	++	T
Cystoseira tamariscifolia	M	++	++
	A	+++	+
	Ch	+	ND
Cystoseira humilis	M	+++	+
	A	+++	ND
	Ch	+	+
Fucus spiralis	M	+++	+
	A	++	-
Rhodophyceae			
Asparagopsis armata	A	+++	+
	Ch	++	-
Bonetia secundiflora	M	++	+
	A	++	T
	Ch	++	-
Corallina elongata	M	++	+
	A	+++	+
	H	++	-
	Ch	+	-
	Aq	++	T
Gelidium latifolium	M	++	+
	A	++	T
Gracilaria multipartita	M	+	-
	A	++	T
	Ch	+	-
Gracilaria verrucosa	M	+++	T
	A	++	T
	Ch	+	-
Halopitys incurvus	M	+++	+
	Ch	+	-
Hypnea musciformis	M	++	T
	A	T	T
Plocamium cartilagineum	M	++	T
	A	++	T
Sphaerococcus coronopifolius	M	++	+
	A	+++	+
	H	++	-
	Ch	+	T

Only few aqueous extracts exhibited antibacterial activity, and the agar disk-diffusion assay may lead to false negative results for aqueous extracts due to the chemical nature of the extracted molecules such as proteins with high molecular weight. Likewise, apolar compounds extracted by hexane did not exhibit significant activity, and the absence of diffusion into agar medium may be responsible for these negative results. It seems that methanol and acetone extract compounds in a polarity range suitable for diffusion into agar.

In order to detect seasonal variations, antibacterial activity was measured for the same species of algae collected during two different periods (March-May and December-January) (table III). Results revealed that the season of harvesting greatly influences the antibacterial activity of the algae. Extracts of algae collected in the Spring were significantly more active than those of algae collected in the Winter. These results were in contrast with those reported by Rao, Parekh (1981) and Vidyavathi, Sridhar (1991), who mentioned that extracts of seaweed collected during the Winter were more active than those from other seasons. Padmakumar, Ayyakkannu (1997) reported that representatives of the class of Chlorophyceae were active throughout the year, in contrast to the Phaeophyceae which showed complete absence of activity in certain seasons, and the Rhodophyceae which showed clear seasonal variations in antimicrobial activity. We never observed total lack of activity during the present study.

CONCLUSION

This study reports the presence of antibacterial compounds in the algae from the Atlantic Coast of Morocco. The antibacterial activity was found predominantly in the class of the Phaeophyceae and methanolic extracts exhibited greater inhibition against Gram-positive bacteria. We showed that many extracts of marine algae collected considerably inhibited the growth of the bacterium *S. aureus*. Moreover we observed significant differences from season to season, with activities generally higher in the Spring.

The growth inhibition of the mycobacterium *M. smegmatis* was also observed with methanolic extracts of several algae. To the best of our knowledge, no previous record of this kind of activity is to be found in the literature.

Disparities reported by different workers for the activity of the same alga may be due to different methods of preservation of algae before extraction, or different solvents used for extraction, to the different susceptibilities among bacterial strains. Such discrepancies in activity could also be explained by geographical variations. Very few screenings for biological activity were done with Moroccan algae extracts. A wide variety of habitats are available for algae in the marine environment with associations with other organisms, such as bacteria. So it is difficult to compare results obtained with algae collected in such different areas as the Moroccan Atlantic. Indian oceanic and Mediterranean coasts. The algal stage of development has to be considered, in this study we observed seasonal variations. The active compound biosynthesis may be linked to the reproduction cycle or the alga. In our ongoing program we will also try to analyse the chemical content of the same alga collected during different stages of development.

Work is in progress to isolate and characterise active compounds and to determine their structures. Since this manuscript was submitted we have published a study of active compounds isolated from *Sphaerococcus coronopifolius* (Etahiri *et al.*, 2001).

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