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Short Communication

Tropical Atlantic marine macroalgae with bioactivity against virulent and antibiotic resistant *Vibrio*

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ABSTRACT. The antibacterial activity of ethanol, methanol, hexane and acetone-based extracts of the macroalgae *Padina gymnospora* (PG), *Hypnea musciformes* (HM), *Ulva fasciata* (UF) and *Caulerpa prolifera* (CP) was investigated. The disk diffusion method was used to evaluate the algae antimicrobial effect against standard strains of *Vibrio parahaemolyticus*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella enterica* and five virulent antibiotic-resistant strains of *V. brasiliensis*, *V. xuii* and *V. navarrensis* (isolated from the hemolymph of *Litopenaeus vannamei*). Ethanol extracts of PG and HM inhibited all *Vibrio* strains. *E. coli* and *P. aeruginosa* were only susceptible to ethanol extracts of PG. Among the methanol extracts, only UF was bioactive, inhibiting *V. navarrensis*. The observed inhibitory effect of ethanol extracts of PG, HM and UF against virulent antibiotic-resistant bacteria suggests these macroalgal species constitute a potential source of bioactive compounds.

Keywords: antibacterial activity, macroalgae, solvent extracts, gram-positive, gram-negative, virulent antibiotic-resistant vibrio.

Macroalgas marinas tropicales atlánticas con actividad biológica contra vibrios virulentos y resistentes a antimicrobianos

RESUMEN. Se investigó la actividad antibacteriana de los extractos de etanol, metanol, hexano y acetona de las macroalgas *Padina gymnospora* (PG), *Hypnea musciformes* (HM), *Ulva fasciata* (UF) y *Caulerpa prolifera* (CP). Se utilizó el método de difusión en disco para evaluar el efecto antimicrobiano de las algas contra cepas patrón de *Vibrio parahaemolyticus*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* y *Salmonella enterica* y cinco cepas virulentas y resistentes a los antimicrobianos: *V. brasiliensis*, *V. xuii* y *V. navarrensis* (aislado de la hemolinfa de *Litopenaeus vannamei*). Los extractos de etanol de PG y HM inhibieron todas las cepas de *Vibrio*. *E. coli* y *P. aeruginosa* solo eran susceptibles a los extractos de etanol de PG. Entre los extractos de metanol, solo UF fue bioactivo, inhibiendo *V. navarrensis*. El efecto antibacteriano de los extractos de etanol de PG, HM y UF contra bacterias virulentas y resistentes a los antimicrobianos sugiere que estas especies de macroalgas constituyen una fuente potencial de compuestos bioactivos.

Palabras clave: actividad antibacteriana, macroalgas, extractos, gram-positivas, gram-negativas, vibrios virulentos resistentes a antibióticos.

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Research on natural compounds as alternatives to synthetic antibiotics is garnering increasing attention in the scientific community (Al-Haj *et al.*, 2010; Vieira *et al.*, 2010). Investigations on bioactive compounds have mostly been focused on phanerogams (Nair & Chanda, 2007; Peixoto *et al.*, 2011), but macroalgae have also been shown to contain compounds capable of inhibiting bacterial growth, including that of well-known human pathogens (Taskin *et al.*, 2007; Kolanjinathan & Stella, 2009).

Macroalgae are multicellular, eukaryotic and autotrophic organisms lacking a specialized vascular system (Pádua *et al.*, 2004). They have traditionally been classified according to their photosynthetic

pigments, although much new taxonomic information has become available. Macroalgae may be found throughout marine and freshwater environments and on soils, rocks, snow and plant surfaces, given appropriate light and moisture conditions (Vidotti & Rollemberg, 2004). As demonstrated by Lima-Filho *et al.* (2002), a number of macroalgae endemic to northeastern Brazil contain bioactive compounds against a broad spectrum of gram-positive and gram-negative bacteria.

The objective of this study was to evaluate the bioactivity of extracts of the marine macroalgae species *Padina gymnospora*, *Hypnea musciformes*, *Ulva fasciata* and *Caulerpa prolifera* against standard strains of *Vibrio parahaemolyticus*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella enterica*, and against antibiotic-resistant vibrios carrying virulence factors.

All strains used in this study were supplied by the microbe bank of the Laboratory of Seafood and Environmental Microbiology (LABOMAR/UFC). The standard strains belonged to five species: *V. parahaemolyticus* ATCC 17802, *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and *Salmonella* IOC (Instituto Oswaldo Cruz). The virulent vibrio strains belonged to three species: *Vibrio brasiliensis* (n = 3), *V. xuii* (n = 1) and *V. navarrensis* (n = 1), originally isolated from the hemolymph of the Pacific white shrimp (*Litopenaeus vannamei*).

The virulent vibrio strains were submitted to detection of proteases (caseinase, elastase and gelatinase), lipase, phospholipase and hemolytic activity. Gelatinase production was tested in tryptone soy agar (TSA, Difco) supplemented with 0.5% gelatin and 1% NaCl (w/v) (Liu *et al.*, 1996). Casein detection was performed using milk agar containing 1% NaCl (Austin *et al.*, 2005). The ability to degrade elastase was tested with a medium composed of nutrient broth and noble agar (Difco) and supplemented with 0.3% elastin (Sigma E1625) (w/v) and 1% NaCl (Rust *et al.*, 1994). The lipase and phospholipase assays were carried out in tubes containing TSA (1% NaCl) enriched with 1% (v/v) Tween 80 and 1% (v/v) yolk emulsion, respectively (Liu *et al.*, 1996). Hemolytic activity was evaluated with Wagatsuma agar (1% NaCl) enriched with 100 mL L^{-1} defibrinated sheep blood suspension at 20% (Wagatsuma, 1968).

In order to determine the antibiotic susceptibility profile, the virulent vibrio strains (*V. brasiliensis* n = 3, *V. xuii* n = 1, *V. navarrensis* n = 1) were challenged with gentamicin (GEN; 10 µg), streptomycin (STR; 10 µg), ampicillin (AMP; 10 µg), penicillin G (PEN; 10 U), imipenem (IPM; 10 µg), cephalothin (CET; 30 µg), ceftriaxone (CRO; 30 µg), chloramphenicol (CHO; 30 µg), aztreonam (ATM; 30 µg), nitrofurantoin (NIT; 300 µg), nalidixic acid (NAL; 30 µg), sulfame-thoxazole-trimethoprim (SUT; 25 µg) and tetracycline (TCY; 30 µg) using the modified disk diffusion method (Bauer *et al.*, 1966). Strains grown in TSA containing 1% NaCl were adjusted to a concentration of 10^8 CFU m L^{-1}, starting with dilution in 1% saline solution (*Vibrio* species) and 0.85% NaCl (*E. coli*, *S. aureus*, *P. aeruginosa* and *S. enterica*) until attaining the 0.5 McFarland turbidity standard. The adjusted strains were inoculated with a swab on Mueller-Hinton agar followed by application of commercially available antibiotic disks (Laborclin). After incubation at 37°C for 24 h, the inhibition halos were measured with a digital caliper (Digmess) and the strains were classified following guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2009).

Macroalgae belonging to the classes Phaeophyta (*Padina gymnospora*–PG), Rhodophyceae (*Hypnea musciformes*–HM) and Chlorophyceae (*Ulva fasciata*–UF and *Caulerpa prolifera*–CP) were collected at Pacheco (a beach location 10 km west of Fortaleza, northeastern Brazil; 3°41'8.6"S, 38°38'5.9"W) at 0.2 m depth during low tide on November 6, 2010. The water temperature at the sampling location was 30°C. The specimens were identified at the Macroalgae Laboratory (LABOMAR/UFC) (Littler & Littler, 2000) and deposited in the herbarium of the same institution under entry numbers 2145 (*H. musciformes*), 2149 (*U. fasciata*), 2152 (*P. gymnospora*) and 2155 (*C. prolifera*). The algae were washed in distilled water to remove epiphytes and debris, dried at 30°C and ground to a homogenous powder for use in the preparation of extracts.

The algae powder (2 g) was submitted to extraction with ethanol, methanol, acetone and hexane at 1:100 (p/v) in a Soxhlet extractor at 78.4, 64.7, 56.0 and 69.0°C, respectively. After 16 h under reflux conditions, the extracts were evaporated to a final concentration of 100 mg mL^{-1}. The extracts were stored at 4°C. Sterilized 5 mm white disks (Laborclin) were soaked with 100 µL extract. Control disks were soaked with 100 µL of each solvent (ethanol, methanol, acetone and hexane).

Strains adjusted to 10^8 CFU mL^{-1} (as described above), were inoculated with swabs on Mueller-Hinton agar (CLSI, 2009). Subsequently, disks soaked in extract were applied in triplicate. A negative control disk (soaked in solvent only) and a positive control disk (soaked in 5 µg ciprofloxacin) were used for each

strain. The plates were incubated at 35°C for 24 h. Inhibition halos ≥ 6 mm were considered evidence of antibacterial activity (Engel *et al.*, 2006).

The *Vibrio* strains isolated from shrimp hemolymph all displayed hemolytic and phospholipolytic activity and a profile compatible with the presence of caseinase. Four of the five strains (80%) were gelatinase-positive, two (40%) presented lipolytic activity against Tween 80, and one (20%) was capable of hydrolyzing elastase (Table 1). Two *Vibrio* strains (40%) were resistant to a single antibiotic (PEN). Three strains (60%) displayed multiple resistance: PEN+CET (n = 1), PEN+CET +AMP (n = 1) and ATM+CRO (n = 1) (Table 1).

The results of the antibiogram test are shown in Table 2. Only ethanol and methanol extracts were bioactive. The greatest inhibition halos were observed for ethanol extracts of PG, ranging from 10.2 ± 1.6 (in *E. coli* culture) to 16.7 ± 2.1 mm (in *V. brasiliensis* culture).

The smallest inhibition halos were observed for ethanol extracts of UF, when compared to extracts of PG or HM. However, UF was the only algal species with antibacterial activity when methanol was used as solvent. In this case, bioactivity was observed only against *V. navarrensis* (average halo size: 11.3 ± 0.8 mm).

PG, HM and UF all displayed vibrocidal activity, especially the first two (Phaeophyta and Rhodophyceae, respectively), which inhibited the growth of all the vibrio species tested. No extract of CP displayed antibacterial activity.

S. aureus and *S. enterica* were resistant to all extracts. The standard strains of *E. coli* and *P. aeruginosa* were susceptible to the ethanol extract of PG only.

Vibrio strains presenting proteases, lipase, phospholipase and β-hemolytic activity may be considered potentially pathogenic (Zhang & Austin, 2005). In view of the presence in our vibrio strains of these markers of virulence along with resistance to one or more commonly used antibiotics, the ability of PG, HM and UF to inhibit these isolates is a very important finding (Table 1), indicating the presence of effective bioactive compounds in the algal species tested.

Shanmughapriya *et al.* (2008) found macroalgae of the classes Phaeophyta, Rhodophyceae and Chlorophyceae to contain possibly lipolytic compounds inhibiting gram-positive and gram-negative bacteria, demonstrating the relevance of investigating the antibacterial activity of algal extracts against pathogens resistant to multiple antibiotics. In fact, according to Watson & Cruz-Rivera (2003), algae may contain a wide range of bactericidal agents, including amino acids, terpenoids, phlorotannins, acrylic acid, phenolic compounds, steroids, haloge-nated ketones, alkanes, cyclic polysulphides and fatty acids.

In our study, ethanol PG extracts presented the broadest spectrum of antibacterial activity, inhibiting all vibrio strains, *E. coli* and *P. aeruginosa* (Table 1). Vallinayagam *et al.* (2009) reported an even broader range of bioactivity for PG extracts prepared from algae collected in India: in addition to inhibiting *Vibrio* and *P. aeruginosa*, extracts were also effective against *S. aureus* and *Salmonella*.

Likewise, ethanol extracts of PG prepared by Manivannan *et al.* (2001) presented bioactivity against not only *V. parahaemolyticus*, *E. coli* and *P. aeruginosa*, matching our findings, but also against *Salmonella*. The authors observed significant inhibition halos when challenging cultures of *V. cholerae*, *V. fluvialis* and *V. splendidus*, confirming the vibriocidal potential of ethanol extracts of PG.

Table 1. Virulence factors and antibacterial resistance profile of *Vibrio* strains isolated from shrimp (*Litopenaeus vannamei*) hemolymph.

Strains	Virulence factor	Resistant to
Vibrio xuii	CAS, GEL, PHOS, HEM	PEN
V. brasiliensis 100	CAS, GEL, PHOS, HEM	PEN, CET
V. brasiliensis 113	CAS, GEL, PHOS, LIP, HEM	PEN, CET, AMP
V. brasiliensis 130	CAS, GEL, PHOS, LIP, HEM	PEN
V. navarrensis	CAS, ELAS, PHOS, HEM	ATM, CRO

CAS: caseinase, GEL: gelatinase, PHOS: phospholipase, HEM: hemolytic activity, LIP: lipase, ELAS: elastase, PEN: penicillin, CET: cephalothin, AMP: ampicillin, ATM: aztreonam, CRO: ceftriaxone.

Table 2. Average inhibition halos of ethanol extracts of the macroalgae *Padina gymnospora* (PG), *Hypnea musciformes* (HM) and *Ulva fasciata* (UF) in cultures of virulent *Vibrio* strains isolated from shrimp (*Litopenaeus vannamei*) hemolymph (SH) and standard ATCC and IOC strains.

Strains	Origin	PG	HM	UF	CIP*
Vibrio xuii	SH	16.5 ± 1.9	11.46 ± 0.5	-	33.00
V. brasiliensis 100	SH	14.9 ± 0.8	8.6 ± 0.6	-	33.36
V. brasiliensis 113	SH	16.7 ± 2.1	10.3 ± 0.3	8.6 ± 0.7	24.06
V. brasiliensis 130	SH	14.7 ± 1.3	9.9 ± 0.5	9.4 ± 0.2	35.25
V. navarrensis	SH	14.9 ± 0.2	14.9 ± 0.2	-	30.18
V. parahaemolyticus	ATCC 17802	14.2 ± 0.8	13.6 ± 0.5	7.9 ± 0.3	23.12
Escherichia coli	ATCC 25922	10.2 ± 1.6	-	-	30.85
Staphylococcus aureus	ATCC 25923	-	-	-	36.43
Pseudomonas aeruginosa	ATCC 27853	10.9 ± 1.4	-	-	33.13
Salmonella enterica	IOC	-	-	-	29.19

* Positive control: 5 µg ciprofloxacin (CIP).

This potential was evidenced in the present study by the inhibition of *V. xuii*, *V. brasiliensis* and *V. navarrensis*, in addition to *V. parahaemolyticus*. In this context, the antibacterial activity of red seaweed (*Gracilaria fisheri*) was reported by Kanjana *et al*. (2011), who verified that ethanol, methanol and chloroform extracts showed activity against a virulent strain of *V. harveyi*.

Extracts prepared with HM and UF were only bioactive against vibrio strains. However, in a study on the bioactive properties of these algal species, Selvin & Lipton (2004) observed a much broader antibacterial spectrum for both UF extracts (inhibiting all the bacterial species tested, including *V. fischeri*, *V. alginolyticus*, *V. harveyi*, *Micrococcus luteus*, *Bacillus cereus*, *B. subtilis*, *E. coli*, *Aeromonas hydrophila*, *P. aeruginosa* and *Aeromonas* sp.) and HM extracts (inhibiting *V. fischeri*, *M. luteus*, *B. cereus*, *B. subtilis*, *A. hydrophila* and *P. aeruginosa*). The antibacterial potential of UF was observed by Selvin *et al*. (2011). These authors suggested that the macroalgae *U. fasciata* may be a source for developing a medicated feed for shrimp disease caused by *Vibrio* and *Aeromonas*.

In our study, none of the 10 strains tested were susceptible to CP extracts. Similar findings were reported by Freile-Pelegrín & Morales (2004) who observed no inhibition halos when challenging strains of *E. coli*, *P. aeruginosa*, *S. aureus* and *Salmonella* with ethanolic CP extracts. Only three of the eight bacterial species tested (*Bacillus subtilis*, *Streptococcus faecalis* and *Micrococcus luteus*) were susceptible to their extracts.

Plaza *et al.* (2010) reported significant variations in antibacterial potential between extracts of the macroalga *Himanthalia elongata* prepared with different solvents. A similar variation was observed in the present study, as none of the four algal species tested (PG, HM, UF and CP) were effective against any of the ten bacterial species when the extracts were prepared with acetone and hexane. This is additionally supported by the absence of antibacterial effects of hexane-based UF and CP extracts upon strains of *E. coli*, *S. aureus*, *P. aeruginosa* and *Salmonella* reported by Lima-Filho *et al.* (2002).

When extracts were prepared with methanol, UF was the only algal species displaying bioactivity, and only one bacterial species was susceptible (*V. navarrensis*). In contrast, Chiheb *et al.* (2009) reported methanol UF extracts to be effective against both *S. aureus* and *E. coli*. However, the bioactive properties of macroalgae are known to vary according to geographic location and season; thus, extracts prepared with the same solvent and the same algal species may differ significantly in antibacterial potential, depending on sampling location and time.

In a study covering 80 species of marine macroalgae, Padmarkumar & Ayyakkannu (1997) demonstrated that while extracts of chlorophycean species remained bioactive all year round, extracts of phaeophycean species lacked antibacterial activity when prepared with algae collected in certain periods.

The bioactivity of extracts of rhodophycean species varied from season to season, but was never completely absent.

In view of the observed inhibitory effect of ethanol extracts of *P. gymnospora*, *H. musciformes* and *U. fasciata* against virulent antibiotic-resistant microorganisms, it may be concluded that these macroalgae represent a potential source of bioactive compounds.

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