JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2017. Vol. **11**(4), p. 1695-1706

Potential Antibacterial Activity of Marine Macroalgae against Pathogens Relevant for Aquaculture and Human Health

Carmen Rizzo[1]*, Giuseppa Genovese[1], Marina Morabito[1], Caterina Faggio[1], Maria Pagano[1], Antonio Spanò[1], Vincenzo Zammuto[1,3], Simona Armeli Minicante[2], Antonio Manghisi[1], Rosalia Maria Cigala[1], Francesco Crea[1], Fabio Marino[1] and Concetta Gugliandolo[1]

[1]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Italy, V.le Ferdinando Stagno d'Alcontres, 31, 98166, Messina,Italy.
[2]National Research Council, ISMAR, Arsenale Castello, 2737/F, 30122 Venezia, Italy.
[3]Department of Earth and Marine Sciences, University of Palermo, via Archirafi 18, Palermo, Italy.

http://dx.doi.org/10.22207/JPAM.11.4.07

(Received: 02 September 2017; accepted: 23 October 2017)

The exploitation of macroalgal biomass, as a source of antibacterial drugs, would result into the valuable transformation of waste into an economic resource. Ethanol and water extracts or polysaccharides, from nine DNA-barcoded macroalgae (2 Chlorophyta, 5 Ochrophyta and 2 Rhodophyta) collected from Italian coastal environments, were screened for antibacterial activity against ten bacterial pathogens with relevance for aquaculture and human health. All extracts have not cytotoxic effects on molluscan digestive gland cells and mammalian red blood cells. As resulted by agar diffusion assays, water extracts showed broader and higher inhibitory activity than ethanol extracts against the tested pathogens. Polysaccharides from *Fucus virsoides* (Ochrophyta) possessed the strongest inhibitory activity against *Aeromonas salmonicida* subsp. *salmonicida* and *Photobacterium damselae* subsp. *damselae*, an emergent pathogen for humans. Polysaccharides from *Undaria pinnatifida* (Ochrophyta) were the most active against *V. harveyi*, the major pathogen responsible for diseases in aquaculture, and also against a multiresistant, clinical strain of *Pseudomonas aeruginosa*. Our results suggest that these polysaccharides could represent a novel source of compounds active against bacterial pathogens relevant for eco-sustainable aquaculture and human health.

Keywords: Algae; Antibacterial activity; Aquaculture; DNA barcoding; Fish pathogens; Algal polysaccharides.

Marine macroalgae represent a great source of biomolecules with a wide spectrum of effects useful in different biotechnological fields, such as in food and textiles industries, biochemistry, pharmacology and in human and veterinary medicine. Green, brown and red algal crude extracts, and their fractioned or purified components, have been reported to exhibit anticoagulant, antibacterial, antiviral, antioxidant and anticancer activities[1-4]. Several studies have reviewed the antimicrobial properties of extracts from marine algae for food and pharmaceutical purposes[5-6]. Since diseases of bacterial origin are cause of considerable economic losses in aquaculture activities, the search for novel metabolites active against pathogens relevant in aquaculture has increased during the recent years[4, 7, 8].

Some species belonging to *Aeromonas* and *Vibrio* genera, which are ubiquitous in freshwater

* To whom all correspondence should be addressed.
Tel.: +39 0906765523; fax: 090 393409;
E-mail: carmen.rizzo@unime.it

and marine environments, have been recognized as pathogenic for a variety of economically important fish and shellfish species, and also for humans, causing enteric pathologies, primary and secondary septicaemia, and wound infections[9, 10]. *A. hydrophila*, frequently isolated from seafood and shellfish, is often associated with diseases of aquatic animals and also is primary or opportunistic pathogen in humans[11]. *A. salmonicida* subsp. *salmonicida* is the causative agents of furunculosis, a bacterial septicaemia of salmonid and turbot[12]. Several vibrios, such as *Listonella anguillarum* (formerly *Vibrio anguillarum*), *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* are responsible for the most common vibriosis in fish and shellfish[13]. On the other hand, *V. cholerae* and non-epidemic vibrios, including *V. parahaemolyticus* and *V. vulnificus,* have been implicated in human diseases associated with the consumption of raw or undercooked shellfish, or the exposure of skin wounds to contaminated fish and waters[14]. *V. harveyi* is considered a serious pathogen of marine fish, the diseases include vasculitis, gastro-enteritis and eye lesions, and of invertebrates, particularly penaeid shrimps and abalone (*Haliotis tuberculata*) of which it is often cause of mortality[15]. Among members of the *Vibrionaceae* family, *Photobacterium damselae* subsp. *piscicida* has been recognized as the causative agent of fish photobacteriosis, and *Photobacterium damselae* subsp. *damselae* has been considered a primary pathogen of a variety of marine animals (including crustaceans, molluscs and cetaceans) and of fish species of economic importance in aquaculture[16]. The isolation of this pathogen from diseased marine fish of new cultured species, suggested that *Ph. damselae* subsp. *damselae* can be considered as an emerging pathogen in marine aquaculture[17]. Most of the reported infections caused by *Ph. damselae* subsp. *damselae* in humans have their primary origin in wounds exposed to salt or brackish water, inflicted during fish and tools handling, that may evolve into necrotizing fasciitis with lethal effects [18 and references therein].

Bioactive compounds from marine algae with antimicrobial activity have been ascribed to a variety of metabolites, such as polysaccharides, polyunsaturated fatty acids, phlorotannins and other phenolic compounds, and carotenoids[1]. Marine algae contain large amounts of polysaccharides, notably cell-wall structural and storage polysaccharides, such as alginates, carrageenans, laminarans, fucoidans and ulvans, accounting from 4% to 76% of dry weight[19-21]. Polysaccharides of algal origin are polymers composed of various monosaccharides linked together by glycosidic bonds, and they have numerous commercial uses, such as stabilisers, thickeners, emulsifiers, food, feed, etc[22].

The variability in the production of antimicrobial compounds by seaweeds, also within the same algal genus, may depend on different ecological factors, such as biogeographical distributions and seasonality, and physiological conditions[1, 4]. Since algal taxonomy is highly convoluted, molecular methods based on DNA barcoding sequencing represent the preferred tools to univocally identify algalspecies with promising applications[23]. Macroalge thrinving in transitional environments, such those of Venice and Cape Peloro coastal lagoons (Italy) produce large amount of biomass, which needs to be periodical removed and treated as a waste, stored in landfills, and finally incinerated.

In order to verify a possible exploitation of algal biomass as a source of antibacterial drugs, with the aim of transforming waste into a valuable resourcein the present study the *in vitro* antibacterial activity of algal extracts obtained from DNA barcoded marine macroalgae was evaluated against bacterial pathogens concomitantly relevant for aquaculture and human health. In consideration of the relevance of the tested pathogens, the cytotoxic effects of extracts were investigated on isolated digestive gland cells of *Mytilus galloprovincialis* and on mammalian red blood cells.

MATERIALS AND METHODS

Sample collection

Samples of Chlorophyta (green algae), Ochrophyta (brown algae) and Rhodophyta (red algae) were collected from different sites in two Italian transitional environments, namely Venice lagoon and lake Ganzirri in Cape Peloro lagoon (Table 1).

Fresh samples were washed with sterile seawater, manually cleaned of epiphytes. From each sample, a part was dried in silica gel and

stored at -20 °C for molecular identification, while the rest was lyophilised for serial ethanol and polysaccharidic extractions.

Algal extracts

Collected samples of red, green and brown macroalgae were washed with clean water and lyophilised (FreeZone® 2.5 Liter Freeze Dry Systems 76705 S, Labconco, USA) and processed to obtain serial extracts of crude ethanol and polysaccharide fractions as detailed in Armeli Minicante *et al.*[3], with some modifications. Each lyophilised sample was soaked in absolute ethanol (about 1:1 v:v) at room temperature for 48 h in a flask and then centrifuged at 4000 g to separate residual thalli. Supernatant was dried with a Rotavapor® at low temperature (35 °C) to preserve volatile compounds from evaporation (crude ethanol extracts).

Subsequently, residual thalli were soaked in distilled water (about 1:3 v:v) at 70 °C for 24 hours in order to extract the polysaccharide fraction. Polysaccharides were precipitated adding an equal volume of absolute ethanol to the mixture and dried at 40 °C overnight.

DNA barcoding

Identification of algal species was performed following standard procedures of DNA barcoding as reported in Saunders and McDevitt[23] and Leliaert et al[24]. Sequences of the mitochondrial COI-5' region were used as marker for red and brown algae, while sequences of the *tuf*A gene were determined for the green alga *Ulva,* and the partial 28S ribosomal gene for the green alga *Chaetomorpha*.

Sequencing reactions were performed by an external company (Macrogen Europe, The Netherlands) or at the Muséum National d'Histoire Naturelle (Paris, France). Obtained DNA barcodes were contrasted with the Barcode of Life Data Systems (BOLD, http://www.boldsystems.org) and with GenBank through the BLAST platform (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to define species attributions. Specimen data and sequences were deposited in BOLD and GenBank.

Antibacterial study

Bacterial strains

Both crude ethanol and polysaccharide extracts were tested against the following pathogenic bacteria concomitantly relevant for aquaculture and human health *Aeromonas hydrophila* (AA5), *Aeromonas salmonicida* subsp. *salmonicida* (13M), *Photobacterium damselae* subsp. *damselae* (Pd), *Pseudomonas aeruginosa* (Pa), *Salmonella* sp. (2sp), *Staphylococcus aureus* (Sa), *Vibrio anguillarum* (Van), *Vibrio cholerae* Non-O1 (Vc), *Vibrio harveyi* (G5) and *Vibrio parahaemolyticus* (L12G). The strains were previously isolated from local marine and brackish environments and identified using standard biochemical and physiological characterization and phylogenetic analysis as reported in Gugliandolo et al[25, 26]. Lab strains are maintained at -20 °C in Tryptone Soy Broth (TSB, Difco) supplemented with 1% (w/v) NaCl (TSB1) and 50% (v/v) glycerol. Clinical strains of *Pseudomonas aeruginosa* (Pa) and *Staphylococcus aureus* (Sa) were chosen on the basis of their strong antibiotic resistance[27].

Antibacterial tests

Antibacterial activity was evaluated by using the standard disk diffusion method (Kirby Bauer test), as accepted by the National Committee for Clinical Laboratory Standards (NCCLS 2000). Strains were overnight grown onto plates of Tryptone Soya Agar (TSA, Oxoid) amended with 1% (w/v) of NaCl (TSA1), for 24 h at 25 °C. Each strain was suspended in 3 ml of 0.9% NaCl solution with turbidity optically comparable to that of the 0.5 McFarland standard (1.5×10^8 bacteria ml^{-1}) and aliquots of each suspension (100 µl) were inoculated onto triplicate plates of TSA1.

Each extract (100 mg, equivalent to 3 g of lyophilised algal sample) was dissolved in 1000 µl of absolute ethanol or sterile distilled water, and 20 µl were applied to sterile filter paper disks (6 mm in diameter, Oxoid). After solvent evaporation, the disks (containing each 2 mg of the extract) were placed onto the inoculated plates. Disks soaked with ethanol and submitted to evaporation were used as negative control, and disks containing chloramphenicol (30 µg) and tetracycline (30 µg) (Difco, Becton Dickinson and Company, USA) were used as positive control. Plates were incubated overnight at 25 °C. The diameter of complete inhibition zones was measured and means and standard deviations (n= 3) were calculated.

Minimum inhibitory concentration (MIC) values were determined for the most active extracts by using the serial dilution assay as accepted by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European

Table 1. List of the algal samples used in this study

Phylum	Voucher	Species	Collection date	Collection site	BOLD accession number
Chlorophyta	PHL672	*Chaetomorpha aerea*	Mar-16	Lake Ganzirri (Messina, Italy)	GRAPP001-17
	SAM029	*Ulva fasciata*	Mar-10	Lake Ganzirri (Messina, Italy)	ITGRE003-11
Ochrophyta	SAM531	*Cystoseira barbata*	May-11	Certosa Island (Venice Lagoon, Italy)	BRAPP004-17
	SAM539	*Sargassum muticum*	May-11	Celestia (Venice Lagoon, Italy)	BRAPP005-17
	SAM099	*Fucus virsoides*	Mar-10	Ottagono S.P. Volta (Venice Lagoon,Italy)	BRAPP002-17
	SAM069	*Undaria pinnatifida*	Mar-10	Celestia (Venice Lagoon, Italy)	BRAPP001-17
	SAM303	*Dictyopteris polypodioides*	Jun-10	Certosa Island (Venice Lagoon, Italy)	BRAPP003-17
Rhodophyta	PHLCB358	*Gracilaria gracilis*	Jan-10	Lake Ganzirri (Messina, Italy)	ITRED096-13
	PHLCB221	*Hypnea cornuta*	Jul-09	Lake Ganzirri (Messina, Italy)	ITRED087-13

Society of Clinical Microbiology and Infectious Diseases[28]. Serial dilutions of each extract (16, 8, 4, 2, 1, 0.5, and 0.25 mg ml^{-1}) were prepared in tubes of TSB1 and then inoculated with suitable aliquots (10 ml) of an overnight culture (comparable to 0.5 McFarland standard) of each strain in TSB1. To confirm the inhibition activity of each extract, 100 ml from all tubes without visible growth were plated onto TSA1, and incubated overnight at 25 °C.

Cytotoxicity assays

Mytilus galloprovincialis specimens (7–8 cm shell length) were collected from the Faro Lake (Messina, Italy). Samples were transferred to laboratory in sterile bags and kept in tanks containing seawater (1100 mOsm/kg; pH= 8.0 ± 0.1; salinity ppm= 37 ± 1) at least for 7 days at 18 ± 1 °C until processing. The digestive gland cells of *M. galloprovincialis* were isolated according to Pagano *et al.*[29, 30], and suspended in saline solution (NaCl 550 mM; KCl 12.5 mM; $MgSO_4$ 8 mM; $CaCl_2$ 4 mM; glucose 10 mM, Hepes 20 mM) at a final concentration of 5 x 10^6 cells ml^{-1}.

In order to define viability, cells were counted by using the Trypan blue exclusion method after 30, 60, 90 and 180 min of incubation, with each algal extract (2 mg ml^{-1}) and in control condition (without algal extract).

The stability of lysosomal membrane was measured by neutral red retention assay (NR), after 30 and 60 min incubation, according to Matozzo *et al.*[31]. Digestive cells of *M. galloprovincialis* incubated in saline solution without algal extracts represented the negative control. All experiments were carried out in triplicate.

Cytotoxic effects were also investigated by incubating mammalian red blood cells for 180 min in physiological solution (0.9% NaCl)[32] containing algal extracts (final concentration of 2 mg ml^{-1}). A control test with mammalian red blood cells incubated in saline solution without algal extracts[33] was performed. Cell viability was determined by Trypan blue exclusion method and the percentage of unstained cells, considered as viable cells, was calculated. All experiments were performed in triplicate.

Determination of carbohydrates in algal extracts

Hydrolysis of polysaccharides was performed with 0.5 N trifluoroacetic acid at 120 °C for 2 h[34]. The standard stock solutions

Table 2. Antibacterial activity of algal crude ethanol (A) and polysaccharides (B) extracts (2 mg/disc) in agar diffusion assay (inhibition zone is expressed in mm) against bacterial pathogens. Values are mean ± standard deviation of three replicates *Ochrophyta*

A) Crude ethanol extract		*Aeromonas hydrophila* (AA5)	*Aeromonas salmonicida* (13M)	*Photobacterium damselae* (Pd)	*Pseudomonas aeruginosa* (Pa)	*Salmonella* sp.(2sp)	*Staphylococcus aureus*(Sa)	*Vibrio anguillarum* (Van)	*Vibrio cholerae* Non-O1 (Vc)	*Vibrio harveyi* (G5)	*Vibrio parahaemolyticus* (L12G)
Ochrophyta	*Cystoseira barbata*	7.0 ± 0.0	-	-	-	-	-	-	10.0 ± 0.0	7.0 ± 0.0	-
Rhodophyta	*Gracilaria gracilis*	7.0 ± 0.0	-	-	-	-	-	-	7.0 ± 0.0	7.0 ± 0.0	-
B) Polysaccharides extract											
Chlorophyta	*Chaetomorpha aerea*	7.0 ± 0.0	-	-	-	-	-	-	-	-	-
	Ulvafasciata	8.0 ± 0.0	10.0 ± 0.0	8.0 ± 0.0	-	-	-	-	8.0 ± 0.0	8.0 ± 0.0	-
Ochrophyta	*Cystoseira barbata*	-	-	-	-	8.0 ± 0.0	-	-	8.0 ± 0.0	-	-
	Dictyopteris polypodioides	-	-	-	-	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0	-	-	-
	Fucus virsoides	9.0 ± 0.0	11.0 ± 0.0	13.0 ± 0.0	-	10.0 ± 0.0	-	-	8.0 ± 0.0	-	9.0 ± 0.0
	Sargassumuticum	-	-	-	-	-	-	-	-	8.0 ± 0.0	-
	Undariapinnatifida	-	-	-	10.0 ± 0.0	-	-	-	12.0 ± 0.0	12.0 ± 0.0	-
Rhodophyta	*Gracilaria gracilis*	7.0 ± 0.0	-	-	-	-	-	-	-	7.0 ± 0.0	-
	Hypnea cornuta	-	-	-	-		-	8.0 ± 0.0	-	-	-
	Chlorampheanicol (30µg)	13 ± 0.0	30 ± 0.0	30 ± 0.3	23 ± 0.0	20 ± 1.5	20 ± 0.0	-	28 ± 0.0	29 ± 0.0	27 ± 0.0
	Tetracycline (30µg)	13 ± 0.0	11 ± 0.0	37 ± 2.8	33 ± 0.0	21 ± 1.4	27 ± 0.0	-	26 ± 0.0	29 ± 0.1	20 ± 0.0

Table 3. Carbohydrates composition of polysaccharide extracts from *Fucus virsoides* and *Undaria pinnatifida*

Carbohydrate	*Fucus virsoides* (ppm)[a]	*Undaria pinnatifida* (ppm)[a]
D-(+)-cellobiose	-	-
D-(-)-fructose	-	295.3
D-(+)-galactose	17.4	57.9
D-(+)-glucose	4.0	-
D-(+)-maltose	-	-
D-mannitol	3.7	51.9
D-(+)-mannose	-	-
D-ribose	-	-
D-(+)-sucrose	-	-
D-(+)-trehalose dihydrate	54.4	269.0
D-(+)-xylose	-	34.1
L-(-)-fucose	512.4	48.5
L-(+)-rhamnose monohydrate	-	344.5

[a] ppm= mg/L (mg of solute/liter of solution)

of carbohydrates were prepared by dissolving appropriate amount of their pure product in ultrapure water. NaOH solution used as eluent was prepared by diluting standard carbonate free concentrated solution. All the carbohydrates (minimum purity 98-99%) and reagents were Fluka or Aldrich products (Sigma-Aldrich Fine Chemicals, Missouri, USA). All the solutions were prepared using grade A glassware and ultrapure water (conductivity <0.1 µS), filtered on Millipore 0.45 µm (Pall Corporation Ann Arbor, Michigan, USA) and then ultrasonicated for 20 min. The chromatographic system was a DX500 ion chromatographic analyser (Dionex Sunnyvale, CA, USA) equipped with Dionex ED40 amperometric cell. Separations were carried out according to the Dionex technical note 21[35] for the analysis of carbohydrates, that are electrocatalytically oxidized at the surface of the gold electrode by application of a positive potential.

The system was equipped with a pre-column (DionexcarboPac PA1, 4 x 50mm) and an analytical column (Dionexcarbopac PA1, 4 x 250 mm). The anion trap (ATC-1 Dionex) and amino trap (P/N 46122 Dionex) were used to remove the dissolved carbonate and the aminoacids.

The eluent was pumped in isocratic condition at 1.0 ml/min; the injection system was a 25 mL Loop. The system was controlled by DionexPeaknet 4.10 chromatographic software. The measurements were carried out in a temperature controlled room at 22 °C.

The carbohydrates taken into account were: D- (+)-cellobiose, D- (-)-fructose; D-(+)-galactose; D- (+)-glucose; D- (+)-maltose; D- (+)-mannitol; D- (+)-mannose; D- (+)-ribose; D- (+)-sucrose; D- (+)-trehalose dehydrate; D-(+)-xylose; L- (+)-fucose L- (+)-rhamnose.

The calibration plots (peak area *vs* carbohydrate concentration) were recovered for a wide concentration range between: $1 \times 10^{-7} \geq$ Cmol $l^{-1r} \geq 1 \times 10^{-4}$. The samples solution, obtained after hydrolysis of the polysaccharides extracted from algae, were previously diluted with ultrapure water in a ratio v/v of 1:10 and 1:150, and then injected.

RESULTS

DNA barcoding

The DNA barcode regions were successfully sequenced for all studied specimens and specific attributions are reported in Table 1 together with accession numbers (BOLD).

Antibacterial activity

Antibacterial activity of both crude ethanol and polysaccharide extracts is reported in Table 2. Algal extracts which did not show any activity against the tested pathogens were not

included.

None of the crude ethanol extracts from Chlorophyta showed antibacterial effects against all the studied bacteria. Among the crude ethanol extracts, those obtained from *Cystoseira barbata* (Ochrophyta) against *V. cholerae* Non-O1 showed the highest activity (inhibition zone of 10.0 mm in diameter), while those from *Gracilaria gracilis* showed only weak antibacterial activity (7.0 ± 0 mm) (Table 2A). Polysaccharides from *Ulva fasciata* (Chlorophyta) were active (10.0 ± 0 mm) against *A. salmonicida* subsp. *salmonicida* (13M), and they showed also weak inhibitory activity against *A. hydrophila* (AA5), *Ph. damselae* subsp. *damselae* (Pd), *V. cholerae* (Vc) and *V. harveyi* (G5) (Table 2B). Polysaccharides from *Dictyopteris polypodioides* (Ochrophyta) resulted active against *V. anguillarum* (Van), *Salmonella* sp. (2sp), and *S. aureus* (Sa). The polysaccharidic extracts from *Fucus virsoides* (Ochrophyta) were active against most of the tested pathogens, and exhibited the highest inhibition activity against *Ph. damselae* subsp. *damselae* (Pd) (inhibition zone of 13.0 ± 0 mm), followed by *A. salmonicida* subsp. *salmonicida* (13M) (11.0 ± 0 mm). Polysaccharides obtained from *F. virsoides* produced an inhibition zone against *A. salmonicida* (13M) equivalent to that of tetracycline (11.0 ± 0 mm) but lower than that of chloramphenicol (30.0 ± 0 mm), used as controls (Table 2B).

Polysaccharides from *Undaria pinnatifida* (Ochrophyta) showed the highest inhibition activity against *V. cholerae* (Vc) and *V. harveyi* (G5) (inhibition zone of 12.0 mm), and they were the only extracts active against *P. aeruginosa* (Pa) (10.0 ± 0 mm).

Within Rhodophyta group, polysaccharides from *Hypnea cornuta* resulted weakly active against *V. anguillarum* (Van) (8.0 ± 0 mm) and those from *G. gracilis* exhibited weak antibacterial activity against *V. harveyi* (G5) (7.0 ± 0 mm).

The minimum inhibitory concentration was determined for polysaccharides obtained from *F. virsoides* and *U. pinnatifida,* as the most active extracts. The MIC values of *F. virsoides* extracts were 2 mg ml^{-1}against *A. salmonicida* subsp. *salmonicida* (13M) and *Ph. damselae* subsp. *damselae* (Pd). Extracts from *U. pinnatifida* showed MIC values of 4 mg ml^{-1}against *P. aeruginosa* (Pa), *V. cholerae* (Vc) and *V. harveyi* (G5).

Cytotoxicity assays

All algal extracts resulted not cytotoxic in the tested conditions. The viability of digestive gland cells of *M. galloprovincialis* and of mammalian red blood cells was on average 85% in all experiments. There were no statistical differences after exposition to different algal extracts (data not shown).

Determination of carbohydrates in algal extracts

The determination of carbohydrates content was performed on the most active extracts obtained from *F. virsoides* and *U. pinnatifida*. After the chromatographic separation, carried out at least in duplicate and taking into account the retention time of the analytes and the dilution of the samples, the qualitative and quantitative results were obtained as reported in Table 3.

In order to verify the correctness of the attribution of the peaks to the carbohydrates investigated, the samples were also spiked with known amounts of carbohydrates and once again analysed; we observed an increase of the peak area proportional to carbohydrate added to sample. This is a clear indication of the correct attribution of the retention time to each carbohydrate, even if the retention time of each analyte was previously determined by injection in the chromatographic system of the pure chemicals (Sigma-Aldrich Fine Chemicals, Missouri, USA) dissolved in ultrapure water.

The preliminary chemical characterization showed that, other than fucose, the most abundant sugars were trehalose and galactose in the polysaccharidic extract from *F. virsoides,* and rhamnose, fructose and trehalose in that from *U. pinnatifida*.

Analysis of the chromatograms highlights the presence in the samples of other components, namely carbohydrates and/or amino sugars, but the unavailability of standards did not allow to perform further qualitative and quantitative determination.

DISCUSSION

Marine algal compounds able to inhibit the growth of bacterial pathogens may represent future alternatives to common antibiotics. The increasing resistance of pathogens to antibiotics represents a priority for exploring and developing effective natural antimicrobial agents with better

potential, good bioavailability, minimal toxicity and less-side effects than antibiotics on humans, farmed organisms and environments.

In this study, both crude ethanol and polysaccharide extracts from nine marine algae (2 Chlorophyta, 5 Ochrophyta and 2 Rhodophyta), collected from different sites in two Italian transitional environments, have been evaluated for their antibacterial effects against pathogens of fish, shellfish and humans. Polysaccharide extracts showed a broader inhibitory activity against the tested pathogens than their ethanol counterparts. Our data revealed that all polysaccharides were active against one or more pathogens, and 67% were active against two or more targeted microorganisms. In contrast, only 22% of crude ethanol extracts from the studied macroalgae were active against one or more pathogens. Extraction protocols, used to test the biological activity of seaweeds, mainly involve different solvents which, depending on their polarity, provide a different efficiency in extracting the active compounds responsible for antimicrobial effects[1]. Moreover, the synthesis of antimicrobial compounds by seaweeds may depend on several factors, including geographical location, environmental factors and algal physiological conditions (active growth or sexual maturity). As reported in our previous study, crude ethanol extracts of *Asparagopsis taxiformis*, collected from the Straits of Messina (Italy) in different sampling periods, exhibited significantly different antimicrobial activity against fish pathogenic bacteria, probably due to a different content of active biomolecules[4]. Indeed, the crude ethanol extracts obtained in late spring showed the broadest activity against the whole panel of studied bacteria, especially against *V. alginolyticus* and *V. vulnificus,* followed by *A. salmonicida* subsp. *salmonicida.*

Seaweed polysaccharide constituents (including alginic acid and alginates, carrageenans and agar, laminarans, fucoidans, ulvans, and derivatives) usually represent the main components of algal cell walls and have important structural functions.

In this work, polysaccharides obtained from the green alga *Ulva fasciata*, and the brown algae *Dictyopteris polypodioides*, *Fucus virsoides* and *Undaria pinnatifida,* were the most active against the studied bacterial pathogens. Extracts from *U. fasciata* showed antibacterial activity against *A. salmonicida* subsp. *salmonicida* (13M), a well-known pathogen able to severely impact salmonid ichthyic stocks[12]. The antimicrobial activity of polysaccharides from *U. fasciata* has not been extensively investigated until now, whereas solvent extracts has been reported active against several Gram-positive and Gram-negative bacteria[36], including *V. parahaemolyticus* and *V. alginolyticus*[37], and have been demonstrated as non-specific defence factors against pathogens of shrimps[38].

Polysaccharides from *Dictyopteris polypodioides* were the most active against *V. anguillarum* (Van), *Salmonella* sp. (2sp) and the multiresistant strain of *S. aureus* (Sa),that is a well-known human pathogen and also an occasionally fish pathogen[39]. Despite its worldwide distribution, very few data are available on the bioactive properties of compounds from *D. polypodioides* and their potential use.

Polysaccharides from *Fucus virsoides* showed inhibitory activity against most of the tested bacteria (6/10), including fish and human pathogens. Interestingly, these extracts were the most active against *A. salmonicida* subsp. *salmonicida*, exhibiting an inhibition zone equivalent to tetracycline used as control, and also against *Ph. damselae* subsp. *damselae* (Pd), a member of *Vibrionaceae* family widely distributed in aquatic environments, and recognized as pathogen of a variety of sea animals. Similarly to its closest related strain *Ph. damselae* subsp. *piscicida*, *Ph. damselae* subsp. *damselae* is also considered resistant to most of the antibiotics used in the treatment of fish pseudo tuberculosis[40-42]. *Ph. damselae* subsp. *damselae* was recently reported as an emergent pathogen for humans, causing infections in wounds exposed to marine or brackish waters and typically associated with fish handling 18 and references therein listed. Necrotizing fasciitis due to *Ph. damselae* subsp. *damselae* have demonstrated to have more serious complications and higher mortality rate in humans than those caused by *V. vulnificus*[43, 44]. More recently, cases of severe wound infection in humans exposed to coastal waters have been referred to both *Ph. damselae* subsp. *damselae* and *V. harveyi*[45, 46]. A preliminary chemical characterization of polysaccharides extracts from *F. virsoides* showed

that the most abundant carbohydrate was fucose, followed by trehalose and galactose, with a global composition close to the commercial fucoidan, a complex sulfated polysaccharide derived from *F. vesiculosus*. Sulfated polysaccharides extracted from different brown algae have been previously evaluated for their activity against both Gram-negative and Gram-positive bacteria, and differences in antimicrobial activities have been mainly related to the sulfate content[47, 48]. The commercially available fucoidan from *F. vesiculosus* has been reported active also against *V. alginolyticus*, however its antibacterial activity was mainly referred to the methanol extracted fraction, rather than to the entire fucoidan content[49].

Polysaccharides from *U. pinnatifida* resulted the most active against *V. cholerae* Non-O1 (Vc) and *V. harveyi* (G5),and were the only extracts active against *P. aeruginosa* (Pa), a multi-resistant strain susceptible only to fosfomycin[27]. Other than fucose, the main components of polysaccharides obtained from *U. pinnatifida* were rhamnose, fructose and trehalose. Despite fucoxanthins, fucoidans and laminarans have been previously reported as active compounds from *U. pinnatifida* against pathogenic bacteria for humans[50-53], no results are actually available against fish pathogens or their potential applications in aquaculture.

The antibacterial activity (evaluated as inhibition zones and minimum inhibitory concentration values) of extracts here reported was overall lower than that of antibiotics used as control, with the only exception of polysaccharides of *F.virsoides* against *A. salmonicida.* Although the exact mode of antimicrobial action of polysaccharides is still not clear, it was proposed that polysaccharides disrupt the bacterial cell wall and cytoplasmic membrane, leading to the dissolution of the protein and the leakage of essential molecules, resulting in cell death[54]. However, further investigations should attempt to purify active compounds from crude extracts and to elucidate their antimicrobial mechanisms.

All the studied polysaccharides have not cytotoxic effects on both molluscan digestive gland cells and mammals red blood cells, indicating a potential use for both aquaculture and human health.

CONCLUSIONS

Our results extend previous observations on the antibacterial activity of macroalgae against pathogens of fish, shellfish and humans. Polysaccharides from nine barcoded macroalgae (2 Chlorophyta, 5 Ochrophyta and 2 Rhodophyta) showed broader and higher inhibitory activity than ethanol extracts against the tested pathogens. Indeed, the ethanol extracts showed a weak inhibitory activity against three of the pathogens (*V. cholerae*, *V. harveyi, A. hydrophila*). Our findings suggest that polysaccharides from the barcoded brown algae *F. virsoides* and *U. pinnatifida* could represent a novel source of antimicrobial compounds against *Ph. damselae* subsp. *damselae* and *V. harveyi*, recently recognized as bacterial pathogens concomitantly relevant for aquaculture and human health. As alternative compounds to common antibiotics for the control of fish infection they could be further investigated also as dietary supplements of farmed fish, in a modern and eco-sustainable aquaculture.The exploitation of algal biomass, as a source of antibacterial drugs, would result into the valuable transformation of waste into an economic resource.

ACKNOWLEDGEMENTS

The research has been carried out under the financial support obtained from the Italian Ministry of University and Research (MIUR), and a grant from the project PON INNOVAQUA (PON02_00451_3362185) to Dr Antonio Spanò. This work was supported by grant from the project FORINNOVAQUA (PON02_00451_3362185/F1) to Dr Carmen Rizzo. Acquisition of molecular data of samples from Venice was carried out at the Service de Systématique Moléculaire (CNRS-UMS 2700), Muséum National d'Histoire Naturelle, Paris, France, courtesy of Dr. Line Le Gall.

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