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Research Article

Chilean benthic species identified as a new source of antibiotic substances

Erwin Strahsburger[1,2], Jessica Pizarro[1], Alex Zúñiga[1], Sergio Ocares[1]
Camila Vallejos[1] & Michael McClelland[2]
[1]Faculty of Renewable Natural Resources, Arturo Prat University, Iquique, Tarapacá, Chile
[2]Department of Microbiology and Molecular Genetics, University of California Irvine
Irvine, CA, United States
Corresponding author: Erwin Strahsburger (estrahsb@unap.cl)

ABSTRACT. Benthic marine organisms are a natural source of bioactive substances with applications in medicine to treat infections, cancer and other diseases. In Chile, this community possesses rich biodiversity that has been scarcely studied as a producer of bioactive substance so far. For that reason, we studied the potential production of antibiotic substances in 28 benthic species that inhabit the coast of the Tarapacá region in northern Chile, and belong to phylum Porifera, Cnidaria, Mollusca, Sipuncula, Annelida, Echinodermata and Chordata. The antibiotic activity was evaluated on ethanol extract obtained from their entire body or dissected tissues and was tested against *Staphylococcus aureus* ATCC 25923 with the diffusion agar method. According to the halo diameter, the antibiotic potency was classified as mild, regular or high. In this way, we could identify 21 benthic species as a producer of antibiotic substances with different antibiotic potency. This activity was found in more than one tissue and with regular or high antibiotic activity in the species; *Acanthopleura echinata*, *Chiton cumingsii*, *Aulacomya atra*, *Fissurella crassa*, *Fissurella latimarginata*, *Luidia magellanica*, *Stichaster striatus*, *Arbacia spatuligera*, and *Loxechinus albus*. The extracts obtained from the entire body and showed regular and high antibiotic activity were from the species; *Phymanthea pluvia*, *Abarenicola affinis*, *Glycera americana*, and *Ophiactis kroyeri*. In conclusion, northern Chile possesses a rich biodiversity of benthic species producer of antibiotic substances, and more research is encouraged to identify these substances with application in medicine.

Keywords: *Staphylococcus aureus*; antimicrobial; organic extract; benthos; northern Chile

INTRODUCTION

The benthic community comprises all organisms living at the sea bottom. Some of them, such as sea urchins, crabs and mollusks are considered an exquisite seafood, while others, such as algae, sponges, holothurians and others are considered a natural source of bioactive compounds with application in medicine (Berlinck *et al*., 2004; Laport *et al*., 2009; Suleria *et al*., 2015; Nalini *et al*., 2018; Puglisi *et al*., 2019). In fact, in ancient traditional medicine, diverse beverage to treat diseases like colds, sore throats, chest infections and tuberculosis were formulated using raw benthic organisms as the main ingredient (Dias *et al*., 2012; Suleria *et al*., 2015; Nalini *et al*., 2018; Prakash *et al*., 2018). To date, benthic species are considered a good source of bioactive compounds and many studies are identifying new antibiotic substances from them (Wagenlehner *et al*., 2016; Sana *et al*., 2017; El Chakhtoura *et al*., 2018; Ogawa *et al*., 2018; Campos *et al*., 2019; Isler *et al*., 2019).

Chilean marine ecosystem is one of the most productive in the world and comprises diverse benthonic species along its coast. This rich biodiversity is due to the presence of different marine currents and environment conditions that harbors a rich trophic chain (Gallardo, *et al*., 1995; Castilla & Fernandez, 1998; Escribano *et al*., 2003; Lee *et al*., 2008; Betti *et al*., 2017; Aguilera *et al*., 2019). However, this richness is contrasted with the few studies about its application as a natural source of bioactive substances. In Valparaiso Bay at central Chile, the southern region and the Antarctica continent, many studies have identified bacteria, algae and benthic organisms as a new source of bioactive compounds with potential application as a news antibiotics, antivirals or antitumoral drugs

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(Henriquez *et al*., 1979; Hayashida-Soiza *et al.*, 2008; Jiménez *et al*., 2011; Sottorff *et al*., 2013; Claverías *et al*., 2015; Arnau *et al*., 2016; Undabarrena *et al*., 2016).

On the other hand, in northern Chile, only two studies have described bioactive compounds in benthic organisms. One identifies four seaweeds species as the producer of secondary metabolites that protect them from mussel's attachment (Pansch *et al*., 2009). Meanwhile, the other study describes a fungus isolated from the marine sponge *Cliona chilensis*, with the ability to produce antimicrobial and antitumor compounds (San-Martin *et al*., 2011). Therefore, the present report aims to increase the knowledge about Chilean benthic marine organisms as a natural source of bioactive compounds, especially in northern Chile. The organic ethanol extract obtained from 28 marine species living along the coast of the Tarapacá region was analyzed on a loan of *Staphylococcus aureus* ATCC 25923. The antibiotic activity observed in many species encourages doing more studies on Chilean benthic organisms as a natural source of bioactive substances with application in medicine.

MATERIALS & METHODS

Collection of benthic organisms

In this study, we included 28 benthic species collected from the intertidal and subtidal zone of coast of Tarapacá region (*ca*. 0-18 m depth), northern Chile (20-21°S) (Table 1). Among 5-10 individuals per each species were collected, preserved in marine water, and transported to the aquaculture facilities of the Faculty of Renewable Natural Resources of Arturo Prat University, Iquique City, Chile. They were maintained in starvation for 24 h in artificial ponds protected from the direct sun exposition and under continuous current of filtered fresh marine water.

Specimen preparation

The benthic species analyzed as an entire body were from phyla Porifera, Cnidaria, Sipuncula, Annelida, Echinodermata and Chordata. They were mechanically cleaned, removing any superficial particles, washed with tap water, and dried at 65°C.

Meanwhile, the dissected marine specimens were from the phyla Echinodermata and Molusca. The entire body was first washed with tap water and then dissected. The specimens of class Asteroidea were dissected in the stomach, ambulacral feet, blind pyloric, gonads, central dermoskeleton and arm dermoskeleton. The specimens of class Echinoidea were dissected in Aristotle's lantern, intestine, spines and gonads differentiated between male and female. Mollusks were dissected in diverse organs according to the species. In general, were obtained the periesophageal ring, foot, radulae, ctenidia, retractor muscles, digestive gland-intestine, mantle, lamellas and gonads, differentiated between male and female. All dissected organs and structures were immediately dried at 65°C.

Ethanol extraction

Each dried sample was ground until obtaining a fine powder and mixed with three volumes of 100% ethanol (Merck-Schuchardt, Germany). The mixture was incubated at 37°C in agitation (150 rpm) for 24 h and finally decanted. The supernatant was filtered with 0.4 µm filters (Fisher Scientific) and incubated at 50°C in a dry-bath (Barnstead/Thermolyne Corp) until it reduces its volume 10 times. The concentrated sample was immediately evaluated with an antibacterial assay or stored at -20°C until further analysis.

Antibacterial assay

The antibacterial assay was performed as described in the literature (Mercado *et al*., 2008) but with modifications. Briefly, the sensitive bacteria *Staphylococcus aureus* ATCC 25923 was culture in Luria Bertani (LB) broth (Difco BD) at 37°C in aerobic conditions. The culture was stopped when it reached an optical density of 0.7 measured at 600 nm in a spectrophotometer (Biomate 5, Thermo Fisher Sci). Then, an aliquot of 0.2 mL was mixed with 5 mL of melted LB soft agar (0.7%), gently mixed and added on the surface of an LB agar plate. After agar solidification (bacterial lawn), a drop of 5 µL from each concentrated ethanol extract was added. A drop of 5 µL of 100% ethanol was included as solvent control. The drops were evaporated under sterile conditions, and then, the plate was incubated at 37°C for 18-24 h. The presence of a halo was representative of inhibition of growing of *S. aureus* strain. The presence of cloudy halo was registered as a mild antibiotic activity (+), a transparent halo with a diameter ≤0.8 mm was registered as regular antibiotic activity (++), and with diameters >0.8 mm as a high antibiotic activity (+++). The antibiotic activity of each active extract was confirmed at least three times.

RESULTS

The extracts from seven benthic species did not show any antibiotic activity (Table 1). The extracts from the other 21 species showed different antibiotic potency among species and body sources utilized (Table 2).

In the phyla Porifera, Cnidaria, Sipucula, Annelida, Echinodermata and Chordata, the most of specimens analyzed as entire body showed a mild antibiotic activi-

Table 1. Analysis of each benthic species collected as a source of antibiotic substances. *The antibiotic activity was assayed on the ethanol extract obtained from the entire organism or tissues from each organism studied, as described in material and methods.

Phylum	Class	Benthic marine specie	
		Antibiotic activity* on *Staphylococcus aureus*	
		Negative	Positive
Porifera	Desmospongiae		*Sponge* sp.
Cnidaria	Anthozoa		*Anemonia alicemartinae*
			Anthothoe chilensis
			Phymactis clematis
			Phymanthea pluvia
Mollusca	Polyplacophora		*Acanthopleura echinata*
			Chiton cumingsii
	Bivalvia		*Aulacomya atra*
	Gastropoda	*Collisella araucana*	*Crepidula dilatata*
		Thais chocolata	*Fissurella latimarginata*
			Fissurella crassa
Sipuncula	Sipunculidea		*Themiste hennahi*
Annelida	Polychaeta		*Abarenicola affinis*
			Glycera americana
			Phragmatopoma moerchi
Echinodermata	Asteroidea	*Heliaster helianthus*	*Luidia magellanica*
		Patiria chilensis	*Stichaster striatus*
	Echinoidea		*Arbacia spatuligera*
			Loxechinus albus
	Holothuroidea	*Athyonidium chilensis*	
		Patallus mollis	
	Ophiuroidea		*Ophiactis kroyeri*
Chordata	Ascidiacea	Ascidiacea sp.	*Pyura chilensis*

ty, while *Phymanthea pluvia* (Cnidaria), *Glycera americana* (Annelida) and *Ophiactis kroyeri* (Echinodermata) showed a regular activity and *Abarenicola affinis* (Annelida) was the most active species (Table 2).

In Mollusca, only *Crepidula dilatata* showed tissues with mild antibiotic activity or no activity. Meanwhile, the other five species have diverse organs where were possible to obtain extracts with regular or high antibiotic activity. The digestive gland-intestine organ was the most frequent source with high activity, while lamellas, radular, periesophageal ring and feet showed a normal antibiotic activity (Table 2). Regarding the gonads as a source of antibiotic substances, the results showed variation among the species. In *Aulacomya atra* its gonads were a source of high and regular activity in female and males, respectively. In *Chiton cumingsii*, *Fissurella latimarginata* (male) and *Fissurella crassa* (male) their gonads were a source of regular antibiotic activity, while in *Acanthopleura echinata*, *Fissurella latimarginata* (female) and *Fissurella crassa* (female) their gonad's extracts did not show antibiotic activity. Other tissues that seem not to be a source of antibiotic substance, according to the methodology described was the retractor muscles of *Aulacomya atra* and the digestive gland-intestine in *Crepidula dilatata* (Table 2).

In the phylum Echinodermata, the species *Arbacia spatuligera*, *Loxechinus albus* and *Luidia magellanica* have external structure like arm dermoskeleton, central dermoskeleton, ambulacral feet and spines that were a good source of antibiotic substances with regular and high antibiotic activity, while in *Stichaster striatus* only the ambulacral feet was a source of regular antibiotic activity (Table 2). While intestine from *A. spatuligera* and *L. albus* were sources of substance with high antibiotic activity. In other internal organs like Aristoteles's lantern in *A. spatuligera* and *L. albus*, and stomach of *L. magellanica* the antibiotic activity extracted showed a regular potency (Table 2). Regarding the presence of antibiotic substances of gonads, the extracts obtained from gonads of female and male of *A. spatuligera* had high and regular antibiotic activity, respectively, while from gonads of *L. albus*, *L. magellanica* and *S. striatus* were inactive.

Table 2. Antibiotic activity detected in the organic extract obtained from the entire body, organs, or structure of each marine organism studied.

Phylum	Species	Antibiotic activity			
		Negative	Mild	Regular	High
Porifera	*Sponge* sp.		Entire body		
Cnidaria	*Anemonia alicemartinae*		Entire body		
	Anthothoe chilensis		Entire body		
	Phymactis clematis		Entire body		
	Phymanthea pluvia			Entire body	
Mollusca	*Acanthopleura echinata*	Gonad (female)	Ctenidia	Periesophageal ring, foot, radular	Digestive gland-intestine
	Chiton cumingsii		Radular	Foot, gonad, digestive gland-intestine	
	Aulacomya atra	Retractor muscles		Mantle and gonad (male), lamellas	Mantle, gonad (female), digestive gland-intestine
	Crepidula dilatata	Gonads, digestive gland-intestine	Foot		
	Fissurella latimarginata	Gonad (female)		Gonad (male), foot	Digestive gland-intestine
	Fissurella crassa	Gonad (female)		Gonad (male), foot	Digestive gland-intestine
Sipuncula	*Themiste hennahi*		Entire body		
Annelida	*Abarenicola affinis*				Entire body
	Glycera americana			Entire body	
	Phragmatopoma moerchi		Entire body		
Echinodermata	*Arbacia spatuligera*			Aristotles's lantern, gonad (male)	Intestine, gonad (female), spines
	Loxechinus albus	Gonads		Aristotles's lantern, spines	Intestine
	Luidia magellanica	Blind pyloric, gonad		Stomach, ambulacral feet, central -dermoskeleton	Arm dermoskeleton
	Stichaster striatus	Stomach, blind pyloric, central dermoskeleton, arm dermoskeleton, gonad		Ambulacral feet	
	Ophiactis kroyeri			Entire body	
Chordata	*Pyura chilensis*		Entire body		

DISCUSSION

In this study, 21 benthic species collected along the coast of the Tarapacá region produced antibiotic substances that were easily extracted with ethanol from diverse sources, showing different antibiotic potency. Regarding the seven benthic species negative for the antibiotic assay, they are members of classes Holothuroidea, Asteroidea, Gastropoda and Ascidiacea, which are known producer of a broad range of bioactive compounds with applications in medicine as anti-angiogenic, anti-hypertension, anticoagulant, antitumor, antimicrobial, and other pharmacological applications (Rahman, 2014). Maybe, the lack of the antibiotic activity observed in this study was due to natural differences among species or because they produce apolar antibiotic substances not extracted with our methodology. The holothurian *Athyonidium chilensis* produce saponins that inhibit the growth of *Staphylococcus aureus* but were extracted with a different methodology using more apolar solvents like dichloromethane and methanol (Sottorff *et al*., 2013). Therefore, other extracted methodologies could be recommended to determine the presence of antibiotic substances in Chilean species of classes Holothuroidea, Asteroidea, Gastropoda and Ascidiacea.

Among the 21 benthic species identified as known producers of antibiotic substances, this is the first time that many of them are reported with this activity and correspond with similar studies in other members of the same class or phylum (Beattie *et al*., 2005; Dias *et al*., 2012; Suleria *et al*., 2015; Mariottini & Grice, 2016;

Nalini *et al*., 2018; Puglisi *et al*., 2019). A detail discussion distinguished by phylum follows.

Porifera

The phylum Porifera includes over 8,600 species distributed across different geographical locations making their comparison complex (Van Soest *et al*., 2012). There is a bacterial biofilm or microbiome associated with the sponge tissue that may synthesize pharmacological substances with application in infections disease and cancer (Laport *et al*., 2009; Indraningrat *et al*., 2016; Beesoo *et al*., 2017). Some of these microbiome bacteria have been isolated from sponge living in Valparaiso Bay at central Chile or Antarctic waters, showing a broad antimicrobial activity and anticancer properties as well (Papaleo *et al*., 2012; Henriquez-Camacho & Losa, 2014; Claverias *et al*., 2015). Therefore, the antibiotic activity observed in the sponge species studied could come from the sponge itself or the associated microbiome. Hence, more research is recommended on these Chilean sponge species to identify the species and the sources of the antibiotic activity.

Cnidarian

Anemones and corals belong to the phylum Cnidaria, and many of them are the producer of peptides and organic compounds with the broad antimicrobial spectrum and extracted with organic solvents like ethanol (Retuerto *et al*., 2007; Mariottini & Grice, 2016). For example, *Stichodactyla mertensii* and *Stichodactyla gigantea* produce antimicrobial compounds easily extracted with ethanol and capable of inhibiting the growth of gram-positive and gram-negative bacteria, and fungi (Thangaraj *et al*., 2011). Meanwhile, on the coast of Costa Rica, the ethanol extract from *Anthopleura nigrescens* showed antibiotic activity against several pathogens including *Pseudomonas aeruginosa*, *Salmonella enterica*, *Escherichia coli*, *Proteus vulgaris* and *Klebsiella oxytoca* (Borbón *et al*., 2016). Regarding the anemones, *Phymanthea pluvia* was the primary source of active antimicrobial extracts among the species studied, and this is described for the first time. In *Phymactis clematis*, the mild antimicrobial activity could be related to the hemolytic peptide Coelenterolysin, which has a similar motif with the antimicrobial peptides Magainin and Dermaseptin (Meinardi *et al*., 1994; Anderluh & Macek, 2002). Meanwhile, for *Anthothoe chilensis* and *Anemonia alicemartinae* this is also the first report describing the antimicrobial activity. In this way, for *Anthothoe chilensis* in addition to the synthesis of highly lethal toxins (TX-1 and Anch TX-2) (Landucci *et al*., 2012), now can we add the synthesis of antibiotic substances that remains to identify.

Mollusca

In this phylum, some species belonging to Polyplacophora, Bivalvia and Gastropoda are known to produce diverse bioactive compounds having possible activity against virus infection (HIV), immune diseases and leukemia, smooth muscle contraction, or nicotine addiction (Hayashi *et al*., 1984; Benkendorff *et al*., 2005; Avila, 2015; Dang *et al*., 2015; Ciavatta *et al*., 2017). In this study, we found six molluscan species producers of antimicrobial substances; *Acanthopleura echinata* and *Chiton cumingsii* (Polyplacophora), *Aulacomya atra* (Bivalvia), and *Crepidula dilatata*, *Fissurella latimarginata*, and *Fissurella crassa* (Gastropoda). Their antibacterial activity was found in diverse organs, but the digestive gland-intestine tissue was the most active. In many marine species, including fish and invertebrates, the digestive gland-intestine tissue synthesizes antimicrobial peptides or enzymes as part of their immune response against infections (Smith *et al*., 2010). Hemocytes present in these organs can also be another source of antimicrobial peptides (Mitta *et al*., 2000).

In chitons, there are not many studies describing antimicrobial compounds (Bekendorf, 2010). Chitons shells harbor the polysaccharide chitin, which can absorb heavy metals and possess antimicrobial activity after alkali conversion in chitosan (Goy *et al*., 2009; Rasti *et al*., 2017; Roy *et al*., 2017). Chitin is insoluble in organic solvents (Roy *et al*., 2017), then maybe other compounds extracted from the foot, gonads and gland-intestine could be related to the antibacterial activity observed in *Chiton cumingsii*.

In Bivalvia, only the mussel specie *Aulacomya atra* was studied. This specie lives along all Chilean coasts and in southern Atlantic. However, is curious that has been extensively studied as indicator of environmental pollution but not as an antibiotic producer (Tapia *et al*., 2010; Pozo *et al*., 2015; Ruiz *et al*., 2018), considering that mussel are natural filter that accumulated high quantities of microorganisms and therefore need of antimicrobial mechanisms to control its microbiome (Leoni *et al*., 2017; Rubiolo *et al*., 2019). In fact, in *A. atra,* high antibacterial activity was observed in its mantle, gonads, digestive gland and intestine. In other Bivalvia species, organs and the entire body have been described with antibacterial activity. In *Perna viridis*, methanol extract obtained from their gills, gastro-intestinal tract and gonads were able of inhibiting the growth of *Acinetobacter baumannii*, *Escherichia coli* and *Pseudomonas aeruginosa*, and this activity were related with a protein of 9.7 kDa (Chandran *et al*., 2009; Kiran *et al*., 2014). While in *Perna erosa*, *Meretrix cast* and *Crassostrea gryphoides*, their methanol extracts obtained from their entire body were shown to have a broad antibacterial and antifungal activity, and seems related with the secretion of antimicrobial peptides (Sharma *et al*., 2009). Therefore, considering all the literature above, there is a high possibility that the antibacterial activity observed in the Chilean mussel *A. atra* may be related to antimicrobial peptides. Hence, more studies have to be addressed to identify this or those antibiotic compounds.

Regarding marine snails (class Gastropoda), the high antibacterial activity observed in the digestive gland-intestine tissue of two *Fissurella* species might be related to hemocyanins. This protein is synthesized by different organs (Arancibia *et al*., 2014; Yao *et al*., 2019), and in *F. latimarginata* is a potent immunomodulator and antitumor molecule on mouse melanoma in comparison with hemocyanins extracted from other gastropods like *Concholepas concholepas* and *Fissurella costata* (Becker *et al*., 2012, 2014; Arancibia *et al*., 2014). Considering that antimicrobial peptides can be derived from hemocyanins (Zhuang *et al*., 2015), it would be interesting to study this protein in both *Fissurella* species.

Sipuncula

Themiste hennahi was the only member of the phylum Sipuncula studied. This report is describing for the first time a mild antibiotic activity in this species, but the chemical nature is still unknown. In similar species, a non-heme respiratory protein called hemerythrins (Alvarez-Carreño *et al*., 2016) plays a biological role in oxygen transport, iron and nitrogen metabolism, heavy metal detoxification and immunomodulation (Sheriff *et al*., 1987; Wittenberg, 1992; Vanin *et al*., 2006; Bailly *et al*., 2008; Stenkamp, 2011; Martín-Durán *et al*., 2013; Coates & Decker, 2016; Alvarez-Carreño *et al*., 2018). However, we observed a direct antibiotic activity instead of an immunomodulatory role, suggesting the presence of other antibiotic substances different from hemerythrins in the analyzed organic extract. Therefore, more studies are encouraged on *T. hennahi* specie to identify the chemical nature of the extracted antimicrobial substances.

Annelida

This paper is the first report describing an antibiotic activity in the annelids species *Abarenicola affinis*, *Glycera americana* and *Phragmatopoma moerchi*. In close related species like *Abarenicola pacifica* and *Arenicola marina,* the synthesis of antimicrobial peptides with a broad antimicrobial spectrum has been reported (Tasiemski *et al*., 2007; Tasiemski, 2008; Lopez *et al*., 2014; Mariottini & Grice, 2016; Vitali, 2018). In the genus *Glycera*, on the contrary, there is not much-related information, because this genus has been studied mainly as a producer of highly toxic neurotoxins that threaten human health rather than of antibiotics (Von Reumont *et al*., 2014). Only *Glycera dibranchiata* was reported as a source of a glycoprotein with a narrow antibiotic spectrum (Anderson & Chain, 1982; Chain & Anderson, 1983). Similarly, the genus *Phragmatopoma* is well known rather as a producer of adhesive molecules than of antibiotic molecules (Basiri *et al*., 2018); therefore, this is the first report describing an antibiotic production in one of its members.

Echinodermata

In the phylum Echinodermata, the analyzed species showed the same antibiotic activity than other Echinodermata species reported in the literature such as *Strongylocentrotus droebachiensis*, *Asterias rubens*, *Cucumaria frondosa* and *Diadema setosum* (Haug *et al*., 2011; Marimuthu *et al*., 2015). These extracts could contain antimicrobial peptides or saponins because both are easily extracted with organic solvents (Andersson *et al*., 1989; Li *et al*., 2008) and are produced by coelomocytes cells or tissue specific cells as part of their immune response against pathogens (Haug *et al*., 2011; Li *et al*., 2015; Coates *et al*., 2018; Nalini *et al*., 2018; Stabili *et al*., 2018). The coelomic cells of *Loxechinus albus* are reported as a producer of antibiotic substances activity against *Escherichia coli* (Pizarro *et al*., 2012), and this study is adding more sources of antibiotic substances in the same species. In the species *Luidia magellanica*, *Stichaster striatus* and *Arbacia spatuligera*, this is the first report in describing this antibiotic activity so far.

Chordata

In the phylum Chordata, only the ascidian species *Pyura chilensis* was analyzed, although many members of this taxonomic group are considered a natural sources of drugs to control depression, anxiety, infections and cancer (Kochanowska-Karamyan & Hamann, 2010; Arumugam *et al*., 2018). This is the first report describing an antimicrobial activity in *P. chilensis*; considering the mild activity observed we suspect the presence of ferreascidin or something similar. Taking into account that ferreascidin is an iron-chelating protein produced by *Pyura stolonifera*, a close specie to *P. chilensis*, and this iron affinity is a common characteristic found in other antimicrobial peptides like cionarin H and cianorin I produced by *Ciona intestinalis* (Dorsett *et al*., 1987; Doshi *et al*., 2011). More studies addressed to identify the chemical nature of the antibiotic compounds extracted and to relate this antibiotic activity with the iron metabolism are recommended.

CONCLUSIONS

This study confirms the richness of Chilean benthic species as a producer of antimicrobial compounds. This property is described for the first time in many of them; being the opportunity to start new research on these benthic species and eventually discover new molecules with application in medicine is emphasized.

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