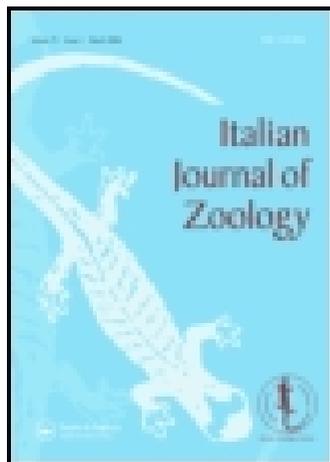


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Antimicrobial peptides from echinoderms as antibiofilm agents: a natural strategy to combat bacterial infections

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Abstract

Increased attention has been focused on marine invertebrates as a source of bioactive molecules for biomedical applications. Many bioactive molecules are part of the innate immune system. Some more recently isolated compounds, mainly from the sea urchin and the sea cucumber, are antimicrobial peptides (AMPs) active against Gram-positive and Gram-negative bacteria, and fungi. In this review we described the most recent studies on AMPs isolated from echinoderms. AMPs are small peptides (< 10 kDa) with cationic charge and amphipathic structure. Recently, it was demonstrated that in the coelomocyte lysates of *Paracentrotus lividus* and *Holothuria tubulosa*, AMPs possess activity against staphylococcal and *Pseudomonas aeruginosa* biofilms. The data shows a great potential for application of AMPs in biotechnology for developing novel therapeutic agents that are either alternative or complementary to conventional antibiotic therapy to combat multi-resistant pathogens.

Keywords: Antimicrobial peptides, echinoderm, *Paracentrotus lividus*, *Holothuria tubulosa*, antibiofilm

Biofilms and antimicrobial peptides

Biofilms are a complex community of microbial cells, enclosed in three-dimensional networks of microbial cells embedded in a self-produced polymeric matrix (slime) (Figure 1). The capacity to organize a biofilm community is present in almost all Gram-negative and Gram-positive bacteria. The process of building a biofilm structure consists, generally, of four stages: (1) adherence of planktonic cells to the abiotic surface or tissue through weak van der Waals forces where the colonists are anchored tightly or irreversibly by pili (Pratt & Kolter 1998) (Figure 1a); (2) the microcolonies' recruitment of other planktonic cells and growth. Normally, they are surrounded by a large amount of extracellular polymeric protective matrix (extracellular polymeric substance: EPS) (Lawrence et al. 1991). The matrix includes a wide variety of proteins, glycoproteins and glycolipids, and, in some cases, surprising amounts of extracellular DNA (e-DNA) (Hall-Stoodley & Stoodley 2009), and can interact with the environment, e.g., by attaching biofilms to surfaces, and through its sorption properties, which allow

for sequestering of dissolved and particulate substances from the environment, providing nutrients for biofilm organisms and influencing predator-prey interactions (Joubert et al. 2006). These components are very important targets for overcoming both biofilms and drug-resistant bacteria (Hancock & Diamond 2000), (Figure 1b). (3) Biofilm maturation involving the development of water channels and specialized zones (Figure 1c), and (4) dispersion of cells and/or parts of the biofilm with subsequent colonization of other locations (Costerton et al. 1981; Donlan & Costerton 2002; Fey 2010; Høiby et al. 2010) (Figure 1d). The architecture of a mature biofilm is variable, ranging from flat, homogeneous layers of cells to highly organized cell clusters with a mushroom-shaped structure containing water-filled channels (Wimpenny et al. 2000).

Although there is great scientific interest and there have been important research advances in the biofilm area, we are still far from being able to control and suppress biofilms. Approximately 80% of human bacterial infections are caused by biofilms (Harro et al.

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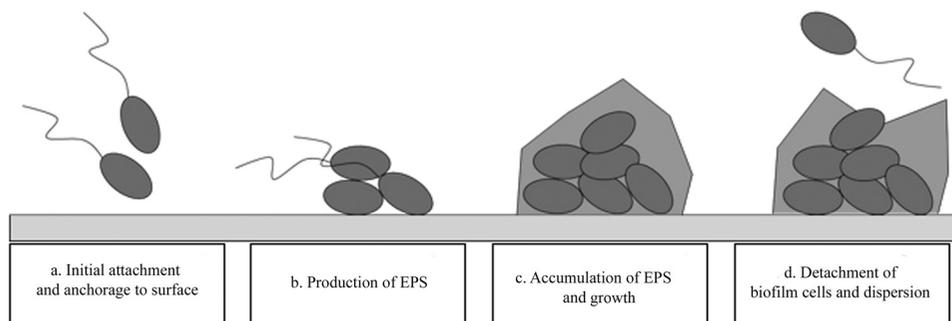


Figure 1. The cycle of biofilm. (a) Attachment of planktonic cells to a surface; (b) recruitment of other planktonic cells and production of extracellular polymeric substance (EPS); (c) growth and development of biofilm through cell division and recruitment; (d) dispersion of bacteria from biofilm.

2010), mainly due to healthcare-associated infections related to the implantation of medical devices, e.g. urinary catheters, intravascular catheters and prosthetic heart valves. As such, microbial adhesion onto surfaces and the subsequent formation of biofilms are critical concerns for many biomedical applications (Donlan & Costerton 2002; Fey 2010). Indeed, the increasing resistance of biofilms to traditional antimicrobial treatments is considered the major cause of dissemination of antibiotic resistance in nosocomial infections (Fey 2010; Spizek et al. 2010).

Antibiotics can be effective against planktonic (free-living) pathogens but are quite often barely effective against the bacteria organized in a community, which can increase antibiotic resistance by 10–1000 fold (Nickel et al. 1985; Evans & Holmes 1987; Gristina et al. 1987; Prosser et al. 1987). It has been estimated that biofilms are associated with nosocomial infections and represent the fourth leading cause of death in the US with ~10% of American hospital patients leading to more than \$5 billion in added medical costs per annum (Wenzel 2007). The bacteria structured in biofilms develop a multifactorial mechanism of resistance to antibiotics (Obst et al. 2006) and there are several factors that contribute to biofilm resistance. The biofilm environment allows a higher frequency of mutation and horizontal gene transmission when compared to planktonic bacteria, which explains the rapid development of antibiotic resistance in biofilms (Ghigo 2001). Within the structure of the biofilm are formed oxygen and nutrient gradients, which cause some form of nutrient limitation. This induces the bacteria to enter into a stationary phase, like dormancy, and under this form bacteria are tolerant to antimicrobials (Brown et al. 1988; Wentland et al. 1996). The resistance can also be due to a general stress response initiated by growth within a biofilm (Brown & Barker 1999). So, bacteria can resist, protecting themselves from the detrimental

effects of heat shock, cold shock, changes in pH and many chemical agents (Hengge-Aronis 1996). The matrix is able to delay antibiotic penetration into the biofilm structure, which contains polymers that bind to antibiotics and hinder their action, and antibiotic-degrading enzymes that deactivate them (Hoyle et al. 1992; Tseng et al. 2013).

The difficulty of successfully treating biofilm-associated infections, and the increasing resistance of microbes to traditional treatments, call for the discovery of compounds with novel mechanisms of action. Most of the antimicrobial products that have been developed are derivatives of compounds that are already known, and hit the same targets, so their action can only be somewhat better. Now, research is drifting towards the discovery of non-traditional sources of antimicrobials, and a series of natural compounds that exhibit antimicrobial activity has been isolated in the past 20 years from many plant, insect and animal species (Roch et al. 1996; Andreu & Rivas 1998; Bulet et al. 1999; Zasloff 2002; Rosetto et al. 2003; Dalla Valle et al. 2013) as defense molecules as humoral parts of the innate immune response (Table I). Animals, in particular, are the most important source of antimicrobial compounds, and several hundreds of antimicrobial peptides have been found in a wide range of invertebrate and vertebrate species. These natural antimicrobial substances, named antimicrobial peptides (AMPs), stand out because they have a much higher hit rate in high-throughput screens than the combinational libraries of traditional antimicrobials. Moreover, natural products are usually much more complex than synthetic products and present scaffolds with viable and biologically validated starting points for the design of chemical libraries (Spizek et al. 2010). According to antimicrobial peptide databases, more than 10,000 AMPs have been discovered to date (Table I).

Table I. List of antimicrobial web databases.

Name	No. of listed antimicrobial peptides	Origin	Web address
 CAMP: Collection of Anti-Microbial Peptides A Comprehensive Antimicrobial Peptide Database	4020	Prokaryotes and eukaryotes	http://www.bicnirrh.res.in/antimicrobial/
YADAMP : yet another db of antimicrobial peptides	2525	Eukaryotes	http://yadamp.unisa.it/default.aspx
 DAMPD: Dragon Antimicrobial Peptide Database Home <input type="checkbox"/> SelectDB <input type="checkbox"/> Tools & Resources <input type="checkbox"/> Acknowledgements <input type="checkbox"/> Contacts Links FAQ Help	1232	Prokaryotes and eukaryotes	http://apps.sanbi.ac.za/dampd/
 The Antimicrobial Peptide Database	2308	Prokaryotes and eukaryotes	http://aps.unmc.edu/AP/main.php
 Antimicrobial Peptides Laboratory DIPARTIMENTO DI SCIENZE DELLA VITA UNIVERSITÀ DEGLI STUDI DI TRIESTE	895	Eukaryotes	http://www.bbcm.units.it/~tossi/amsdb.html
 BACTIBASE database dedicated to bacteriocins	177	Bacteriocin	http://bactibase.pfba-lab-tun.org/statistics.php
 DEFENSINS Knowledgebase A collaboration between Bioinformatics Institute and the Singapore Eye Research Institute	363	Defensin	http://defensins.bii.a-star.edu.sg/
 PhytAMP a database dedicated to plant antimicrobial peptides	271	Plant	http://phytamp.pfba-lab-tun.org/main.php

The largest group of AMPs is that of cationic molecules, which are widely distributed in animals and plants. They have a small molecular size with no more than 100 amino acids and a molecular weight < 10 kDa. They differ considerably in amino acid sequence and structural conformation, and most of them are positively charged, showing a net positive charge of +2 to +9 due to an excess number of basic amino acids like arginine or lysin, with 50% or more of the amino acids hydrophobic and forming a hydrophobic face opposite to a hydrophilic one (Brogden 2005; Hancock & Sahl 2006). On the basis of their structural features, cationic AMPs can be divided into linear peptides forming α -helical structures, cysteine-rich open-ended peptides containing single or several disulfide bridges and molecules rich in specific amino acids such as proline, glycine or histidine. These chemo-physical characteristics allow these peptides to be soluble in water but react simultaneously with the hydrophobic layer of microbial membranes. Such peptides are found in all species of life including bacteria, fungi, plants, insects, birds, crustaceans, amphibians and

mammals. A single animal can contain different classes of peptides and a number of variants in a given class.

The mode of action of AMPs has rarely been addressed and is therefore not yet understood (Brogden 2005). It is generally agreed that the prevalent mechanism of action of the AMPs is due to their ability to permeabilize and/or to form pores within the cytoplasmic membranes. AMPs are initially recruited on the microbial surface through electrostatic interaction between the cationic portion of the peptides and the negatively charged microbial cell walls and/or membranes. Bacterial pathogens' membranes are composed predominantly of phosphatidylglycerol, cardiolipin or phosphatidylserine and tend to be highly electronegative. Studies support a non-receptor type interaction for antimicrobial peptides with most pathogen membranes (Bessalle et al. 1990; Wade et al. 1990). When the peptide/lipid ratio increases, the peptides start forming multimers or self-associating on top of the membrane. When the peptides reach a high concentration, they orientate perpendicularly and insert into the bilayer,

thereby interfering with membrane integrity. The microorganisms are then destroyed via either membrane destabilization and/or pore formation (Brogden 2005; Yount et al. 2006), or through interference with several essential metabolic functions, such as protein, nucleic acid and cell wall syntheses acting on nucleic acids and/or enzymatic proteins, leading to bacterial cell death (Kamysz et al. 2003; Brogden 2005; Yount et al. 2006; Hale & Hancock 2007; Nicolas 2009). Moreover, it appears that many AMPs may be multifunctional microbicides, acting simultaneously at the cell membrane and internal sites (Yount et al. 2006).

Recent research has shown that AMPs also have a high potential for inhibiting the formation of or destroying biofilm. In fact, they can act at several stages of biofilm formation and with different mechanisms of action: they may minimize the initial adhesion of microbial cells to abiotic surfaces by altering the adhesive features of abiotic surfaces, reducing flagellum-dependent swimming motility, stimulating twitching motility, a type of surface motility that promotes the disassembly of biofilm structures or by binding to microbial surfaces via electrostatic interactions; they may prevent biofilm maturation by killing the early surface colonizers, or by inhibiting quorum sensing (QS) – that is, the communication system used by many bacteria to build a biofilm. QS is a system composed of small molecules that control collective behaviors, such as bioluminescence, virulence factor production and biofilm formation (Spoering & Gilmore 2006; Horswill et al. 2007; Picioareanu et al. 2007; Huang et al. 2010; Brogden & Brogden 2011; de la Fuente-Nunez et al. 2012).

AMPs are produced by living organisms throughout the bacteria and animal kingdoms, including also fungi and plants (O’Keefe 2001; Rodriguez et al. 2002; Zasloff 2002; Castro & Fontes 2005; Cotter et al. 2005; Riley & Chavan 2007; Strominger 2009; da Rocha Pitta et al. 2010). Currently, more than 4000 peptides have been isolated and characterized from tissues and organisms, and have been listed in the main databases or in journal publications (Thomas et al. 2010).

In invertebrates, AMPs are ubiquitously distributed, found especially in hemolymph and in tissues such as epidermis, gut and respiratory organs where exposure to pathogenic microorganisms is most likely to occur, expressed constitutively or in response to a pathogen stimulus. The AMPs defend the organism not only through their ability to kill bacteria, but it has been shown that they have anti-tumor effects and mitogenic activity and, most importantly, participate in immunoregulatory

mechanisms by modulating signal transduction and cytokine production and/or release (Hancock & Diamond 2000; Zasloff 2002; Bals & Wilson 2003; Kamysz et al. 2003; Bowdish et al. 2005; Brown & Hancock 2006; Hancock et al. 2006; Yount et al. 2006; Easton et al. 2009; Lai & Gallo 2009; Guani-Guerra et al. 2010).

The purpose of this review will be to present the most recent data on microbial antibiofilm peptides isolated and characterized in the phylum Echinodermata. In particular we will focus on their structure and biological functions, and on their potential application as antimicrobial and antibiofilm agents to combat human pathogens.

Antimicrobial and antibiofilm peptides in echinoderms

Echinoderms are deuterostome invertebrates, an ancient group of marine invertebrates that live in both the intertidal and deep-sea benthos, composed of approximately 7000 extant species including sea stars (asteroids), sea urchins (echinoids), sea cucumbers (holothurians), brittle stars (ophiuroids) and sea lilies (crinoids). Because many species live in or near the coastal or estuary waters overloaded with infectious organisms – such as viruses, bacteria, fungi and other parasites – they are exposed to a broad variety of challenges to their self-integrity. They have evolved efficient defense strategies in order to survive in these high-impact environments.

Echinoderms, similarly to other invertebrates, do not possess a specific immune response. Therefore, they ensure the protection of their organism and its homeostasis using natural immunity responses.

The echinoderm humoral immune response consists of the production of proteins that are sequestered within the cells, having hemolytic and hemagglutinating properties (Canicatti 1987, 1988, 1992; Arizza et al. 2007), and also includes the synthesis of AMPs that are secreted from the coelomocyte.

Beauregard et al. (2001) discovered, for the first time in echinoderms, the presence of AMPs, a peptide of ~ 6 kDa active against Gram-positive and Gram-negative bacteria, in the coelomic fluid of the sea cucumber *Cucumaria frondosa* (Gunnerus, 1767). Since 2001, the number of such discovered molecules has increased steadily. Stabili et al. (1996) found in coelomic fluid and in coelomocytes of *Paracentrotus lividus* (Lamarck, 1816) the effector cells of immunity, a peptide of 60 kDa that was able to inhibit the growth of *Vibrio alginolyticus*. Other antimicrobial activity was found in the jelly coat, in seminal plasma

(Stabili & Canicatti 1994) and in the larval lysate of the same species (Stabili et al. 1994). In some cases, antimicrobial proteins were also found in the gastrointestinal organs and in the eggs of *Strongylocentrotus droebachiensis* (Müller, 1776) and *Asterias rubens* (Linnaeus, 1758) (Haug et al. 2002; Li et al. 2008). Many AMPs are derived from larger proteins that could be enzymatically digested and produce active fragments. This has been demonstrated by (Ghanbari et al. 2012). In fact, digesting the tissues of sea cucumber *Actinopyga lecanora* (Jaeger, 1833) with bromelain, peptides with inhibitory activities against *Pseudomonas* sp., *P. aeruginosa*, and *Escherichia coli*, respectively, were obtained and one papain-digested fraction showed antibacterial activity against *Staphylococcus aureus*. Maltseva et al. (2007) found that among the AMPs isolated in the starfish *Asterias rubens*, two peptides were part of the histone molecule H2A. Maltseva and co-workers also found that two other peptides were fragments of actin, while one peptide was a fragment of filamin A. Gowda et al. (2008) showed that an agglutinin that can agglutinate Gram-positive and Gram-negative bacteria exhibited strong antibacterial activity both under in vivo and in vitro conditions. Defensin-like peptides were isolated by Ng et al. (2013) in *Strongylocentrotus droebachiensis*. In a recent study, Li et al. (2010) showed, in *S. droebachiensis*, an AMP heterodimer structure named centrocin. Centrocins possess a strong activity, not only against Gram-positive and Gram-negative bacteria, but also against fungi and yeasts.

The simultaneous presence of diverse AMPs found in the same echinoderms as *S. droebachiensis*, probably acting in synergy or complementary to each other, may provide the organisms with an extended defense against a wide range of pathogenic microorganisms. Such interactions have been reported, at least in vitro, between different AMPs isolated from the horseshoe crab (Iwanaga et al. 1998) and the oyster, *Crassostrea gigas* (Thunberg, 1793) (Gueguen et al. 2009).

Recently, our research group has found novel cationic peptides in the echinoderm species *Paracentrotus lividus* and in *Holothuria tubulosa* (Gmelin, 1788). They were isolated from coelomocyte lysate supernatant and showed good activity in preventing the biofilm formation of important pathogens involved in human and animal diseases, like staphylococcal or *P. aeruginosa* strains (Schillaci et al. 2010, 2013).

The antimicrobial peptides were discovered in a protein fraction at low molecular weight (< 5 kDa) from acid extract of coelomocytes. *P. lividus* showed a peptide, Paracentrin 1, of 1251.7, the peptide

belonging to a segment (9–19) of a β -thymosin, a ubiquitous peptide that exerts several biological effects such as the induction of metalloproteinases, chemotaxis, angiogenesis and inhibition of inflammation (Huff et al. 2001). The β thymosins are a family of highly conserved polar 5-kDa peptides originally thought to be thymic hormones. They are present at high concentrations in almost every cell from vertebrate phyla and have several biological functions due to direct and indirect effects on the actin cytoskeleton. β -Thymosin is also described as one of the AMPs of platelets from animals, including human beings (Tang et al. 2002). There is little information about the function of thymosins in invertebrates, but their presence has been reported in marine invertebrates (Safer & Chowrashi 1997; Saelee et al. 2013) and in insects where they are up-regulated by microbial infections (Zhang et al. 2011). *H. tubulosa* possessed two peptides, Holothuroidin 1 and Holothuroidin 2, whose molecular weights are respectively 1389.5 and 1547.6 Da (Schillaci et al. 2013).

The peptides of both species showed a similarity with other antimicrobial peptides produced by different organisms. Indeed, Paracentrin 1 had a amino acid sequence similarity of 38.46% with Jelleine-III, a short peptide presenting a broad spectrum of activity against Gram-positive and Gram-negative bacteria, and also against yeasts present in the royal jelly produced by *Apis mellifera* (Linnaeus, 1758) worker bees (Fontana et al. 2004); both Holothuroidin 1 and Holothuroidin 2 showed an amino acid sequence similarity $\geq 35\%$ respectively with protonectins, peptides present in the venom of the neotropical social wasp *Agelaia pallipes* (Lepeletier, 1836), with a potent antimicrobial action against both Gram-positive and Gram-negative bacteria (Mendes et al. 2004) and signiferins, a naturally occurring cationic peptide produced by an Australian frog, *Crinia signifera* (Girard, 1853), that showed a wide spectrum of activity against Gram-positive and Gram-negative bacteria including *Bacillus cereus*, *Enterococcus faecalis*, *Lactococcus lactis*, *Listeria innocua*, *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus uberis* (Maselli et al. 2004).

The peptides of both species had α -helix structures with a considerable amphipathic character, with the polar and mainly cationic residues segregated on one polar face and the hydrophobic or nonpolar residues on the opposite, apolar face. (Figure 2). They were cationic peptides with a net charge of +0.9, with a total hydrophobic ratio and deduced pI ranging respectively from 36.36 and 8.72 for Paracentrin 1 to 42.86 and 7.56 for both Holothuroidins (Table II).

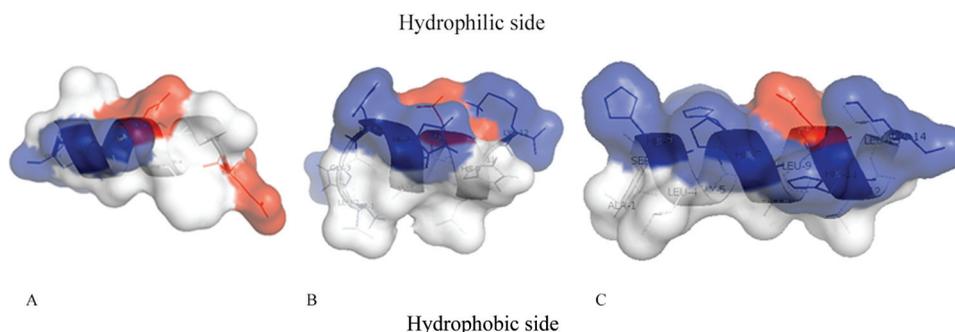


Figure 2. A ribbon representation of (A) Paracentrin 1, (B) Holothuroidin 1 and (C) Holothuroidin 2. The amphipathic nature of the peptide is shown in this representation with the hydrophilic side above and the hydrophobic side below the polypeptide backbone. The potential surface is superimposed. Color code: acidic residues in red, basic residues in blue and hydrophobic residues in white.

Table II. Chemical-physical characteristics and amino acid sequences of echinoderm antibiofilm peptides.

Peptides	Sequence, and hydrophobic amino acids on the sameface (underlined)	Molecular weight (Da)	Net charge (pH 7.0)	Total hydrophobic ratio (%)	pI
Paracentrin 1	EVASF <u>DKSKLK</u>	1251.4	+0.9	36.36	8.72
Holothuroidin 1	HLGHH <u>ALDHL</u> LLK	1389.5	+0.9	41.67	7.56
Holothuroidin 2	ASHLGH <u>HALDHL</u> LLK	1547.6	+0.9	42.86	7.56

The activity of synthetic peptides constructed from the sequences indicated by the tandem mass spectrometry (MS/MS) data were active against free-living (planktonic) Gram-positive and Gram-negative pathogens as *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (Table III) (Schillaci et al. 2010).

The three echinoderm antimicrobial peptides were also active to combat the biofilm formation at sub-minimum inhibitory concentration (MIC) concentrations. Indeed, Paracentrin 1 was able to inhibit either the formation of a young biofilm (6-h old) of *S. epidermidis* 1457 or the formation of a mature biofilm (24 h old) of the same clinical strain Figure 3 (Schillaci et al. 2010). The antimicrobial peptides from *H. tubulosa* were able to inhibit the biofilm formation of two staphylococcal reference strains, *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 35984 (Schillaci et al. 2013).

We observed that synthetic Paracentrin 1, Holothuroidin 1 and Holothuroidin 2 did not show, in phosphate buffered saline (PBS), hemolytic activity, probably because the high ionic strength of PBS allows negatively charged sialic acid present on the erythrocyte membrane to neutralize the peptides. On the basis of this evidence, they could be classified as peptide antibiotics (Saberwal & Nagaraj 1994).

Conclusions

AMPs are evolutionarily ancient defensive molecules. Their extraordinary distribution in all kingdoms, within both unicellular and multicellular organisms, suggests that they have a key and fundamental role in the biology of organisms that probably has evolved through positive selection (Tennessee

Table III. Antimicrobial activity (MIC) of < 5-kDa peptide fraction from celomocytes lysate supernatant (Paracentrin 1) or synthetic peptides (Holothuroidin 1 and 2). Values expressed in mg/mL.

Strain	Paracentrin 1	Holothuroidin 1	Holothuroidin 2
<i>Staphylococcus aureus</i> ATCC 29213	126.8	> 12.5	12.5
<i>Staphylococcus aureus</i> ATCC 25923	63.4	12.5	12.5
<i>Staphylococcus aureus</i> ATCC 43866	63.4	nd	nd
<i>Staphylococcus aureus</i> ATCC 6538	nd	12.5	12.5
<i>Staphylococcus epidermidis</i> ATCC 35984	nd	12.5	12.5
<i>Enterococcus faecalis</i> ATCC 29212	nd	>12.5	12.5
<i>Pseudomonas aeruginosa</i> ATCC 15442	nd	12.5	12.5

(Schillaci et al. 2010)

(Schillaci et al. 2013)

nd = not determined.

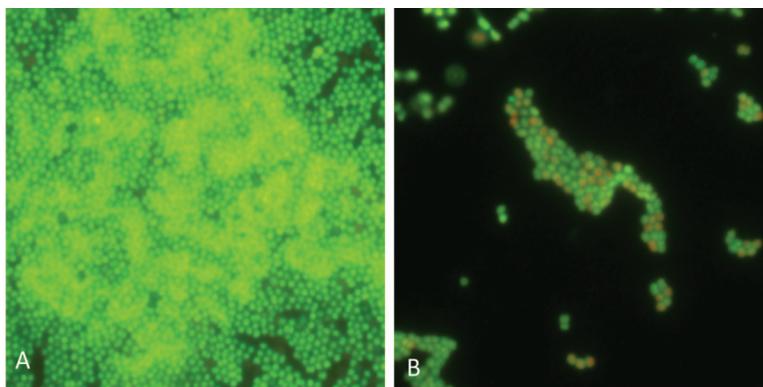


Figure 3. Anti-biofilm activity of Paracentrin 1 against *Staphylococcus epidermidis*. (A) Control; (B) biofilm treated with sub-minimum inhibitory concentration of Paracentrin 1.

2005; Viljakainen & Pamilo 2008; Fernandes et al. 2010).

AMPs from marine invertebrates could play a complementary but critical role in helping acquired immunity in vertebrates to combat bacterial infections that normally confound and even suppress the immune system with their sophisticated and multiantigenic cycle of life (Chiu et al. 2013).

AMPs from marine invertebrates can be applied in biotechnology and in medicine. These natural compounds constitute potential candidates for the development of alternative strategies to prevent and treat bacterial infections, including biofilm-associated infections that are particularly resistant to conventional antibiotics.

In conclusion, the discovery of novel peptides in echinoderms represents a good starting point to design new synthetic derivatives with modified chemical-physical properties, with the aim being to improve their antimicrobial activity against pathogens, and their pharmaceutical potential (Huang et al. 2010; Brogden & Brogden 2011).

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