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The anti-biofilm effects of sponge *(Callyspongia sp.)* and two sea anemones *(Zoanthus sansibaricus and Cerianthus lloydii)* collected from the Persian Gulf

Homa Hamayeli, M.Sc.[1], Mehdi Hassanshahian, Ph.D.[2], Mahasti Mohammadi, M.Sc. [3]

1- MSc of Microbiology, Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran

2- Associate Professor of Microbiology, Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran (Corresponding author; E-mail: mshahi@uk.ac.ir)

3- MSc of Microbiology, Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran

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Abstract

Background: Sponges and sea anemones do not have specialized defense organs. Instead, they rival harmful microorganisms by producing certain compounds. These compounds can also be useful against some human pathogens. This study aimed to investigate the antimicrobial effects of bioactive products from these marine animals.

Methods: Two species of sea anemone *(Zoanthus sansibaricus* and *Cerianthus lloydii*) and one species of sponge (*Callyspongia sp.*) were collected at the Persian Gulf. The active metabolites of these two marine animals were extracted by methanol and dichloromethane solvents. The antimicrobial activity of each extract was performed against six human pathogenic bacteria including: *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Bacillus cereus*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Staphylococcus aureus*, using disk diffusion and agar well plate methods. The MIC and MBC were determined. The inhibitory effect of these extracts on biofilm formation was also studied.

Results: The effect of sponge extracts against planktonic forms of bacteria showed that the most sensitive bacteria to *Callyspongia sp.* extracts were *K. pneumoniae* and *S. aureus*. However, *C. lloydii* did not have any inhibitory effect on *K. pneumoniae*. The results of this study confirmed that both sponge and sea anemones extracts had sufficient effects against biofilm formation of pathogenic bacteria. However, *Callyspongia sp.* extracts had the lowest inhibitory effect against biofilm formation of *P. aeruginosa*. The highest inhibitory effect was observed on biofilm formation of *K. pneumoniae*.

Conclusion: The *Callyspongia sp* sponge extract (Dichloromethane: Methanol) has an excellent antimicrobial effect against six pathogenic bacteria in planktonic and biofilm forms. There was a direct correlation between the increase in the concentration of sea anemones extracts and the inhibitory effect of biofilm formation.

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Introduction

Marine natural products have a vast range of biological activities and play influential roles in the development of drugs for the treatment of human diseases. A large number of unique compounds found from Porifera (often sponge) and Cnidaria produce more bioactive compounds than other marine organisms (1).

Organisms that lack external defense tools can develop chemical defense strategies especially through the production of secondary metabolites with antibiotic or repellent effects. Secondary metabolites from marine organisms have proven to be an exceptionally rich source of small molecules with pharmacological activities possibly beneficial to human health (2).

The effective drug were obtain from marine invertebrates, especially marine sponges being the organisms most widely investigated. In fact, approximately 16,000 marine natural compounds have been isolated from marine organisms and more than 5300 of these compounds were isolated from marine sponges (2).

Among marine organisms, cnidarians are an ancient group of venomous animals that these organisms can concentrated toxins and delivery it. Members of the Anthozoa class are good candidate for drug discovery. It has been guessed that at least 250 compounds have been purified from animals of this class (3).

Cnidaria is an ecologically valuable phylum. It includes about 9400 species, of which 68% are members of the Anthozoa class. Generally anthozoans need to protect themselves against the fatal or debilitating outcomes of the microbial or parasitic invasion. Sea anemones produce multiple biologically active polypeptides (4).

Biofilm plays an important role in the virulence of bacteria. There are many benefits for microorganisms to form biofilms. They provide an enclosed surface space which is occupied and can provide a level of stability in the growth environment. Microbial biofilms are associated with a lot of persistent infections which respond inadequately to antibiotic therapy and can endure host immune response (5).

Access to antiseptics and antibiotics for health care practitioners has been identified, optimized, and commercialized using planktonic methods to assay antimicrobial activities. It is not unusual that agents developed for planktonic targets perform sub-optimally against biofilm targets. One of the first recommendations that can be made is simply to use biofilm-based methods in the exploration for new drugs (6).

Marine organisms are of particular interest and have not been thoroughly explored as sources of bioactive molecules for the development of innovative chemotherapeutics (7).

The purpose of this research was to investigate the antibacterial effect of the sponge extract (*Callyspongia sp.)* and two species of sea anemone extracts (*Z. sansibaricus* and *C. lloydii)* against six human pathogens and their inhibitory effect on the microbial biofilm.

Material and Methods

Collection of marine sponge and sea anemones

Marine sponges were collected at the Persian Gulf in 2015. These sponges were gathered from a depth of 15

meters in Lesser Tunb islands. Sea anemone *Z. sansibaricus* was collected in the rocky intertidal zone in Qeshm island, and sea anemone *C. lloydii was collected in the* depth of 1-5 meters in Bandar Abbas beach (Figure 1). The collection was repeated three times. They were transported alive in sea water to the laboratory and maintained at 4ºC in the refrigerator before extraction. The collected sponges were identified according to the protocol of Hooper and Boury-Esnault (8,9).

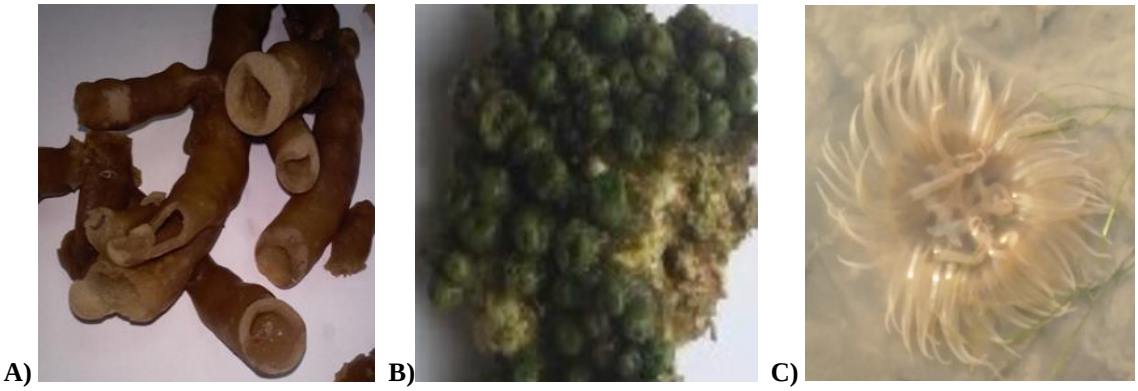


Figure 1. Pictures of the sea anemones and the sponges investigated in this study: A) Sponge Callyspongia sp. B) Sea anemone Z. sansibaricus C) Sea anemone C. lloydii

Preparation of sponge and sea anemone extracts

Based on the protocol, we extracted 100 g of each freeze-dried samples (sponge and sea anemones). The sponge *Callyspongia sp*., immersed into a polar and nonpolar solution with dichloromethane (DCM): methanol (MeOH) (1:1 v/v) and the sea anemones *Z. sansibaricus* and *C. lloydii* were immersed in methanol for two days. Then, the obtained extract was filtrated through Whatman No. 1 filter paper and was concentrated by incubator at 40°C for 24 h. The filtrate was placed into the incubator at 40°C for 24 h to remove the solvent. The concentrated extracts were applied for antimicrobial activity against pathogenic bacteria.

Bacteria

Six antibiotic resistant pathogenic bacteria were used in this research. They included *Pseudomonas aeruginosa* (ATCC 27853), *Acinetobacter baumannii* (ATCC 19606), *Bacillus cereus* (ATCC 14579), *Klebsiella pneumoniae* (ATCC 700603), *Escherichia coli* (ATCC 35218), *and Staphylococcus aureus* (ATCC 25923).

Disk diffusion method

Antibacterial activities of each extract (two sea anemones and one sponge) were assayed by the standard disc diffusion method. This test was done based on the Kirby-Baer disk-sensitive test protocol. The turbidity of bacterial culture was equal to the McFarland (0.5) turbidimeter (10^8 CFU/ml). The suspension were culture by sterile swap into MHA plates. Then, 100 mg/mL concentration of each extract with a solution prepared from a blank disc (6 mm) was placed into each of these concentrations for 1 hour. The disc was put for 30 min at room temperature and transferred on top of the

plate. Penicillin and Chloramphenicol were used as positive control. After overnight incubation, the zone of inhibition (ZOI) of each disc was calculated in millimeter and the measurement was performed in triplicate for each sponge extract (10).

Antibacterial assay of marine organism extracts by agar well plate method

The dissemination of each extract directly into agar well was performed to detect the antibacterial activity. The overnight culture of bacteria was grown by spread method on MHA plates. Some wells (8 mm diameter and about 1.5 cm wide) were created in each MHA plate by a sterile blade. The extract of each marine organism was provided with 100 mg/mL concentration of each extract with a solution. Then, 40 µl of concentration from the each extract were inoculated by a sterile syringe into the constructed wells and remained to diffuse at 30 °C for 2 h. Also, inoculums without marine organism extracts were designed as a negative control. The MHA plates were incubated at 37°C for 24 h and ZOI was measured. This experiment was carried out three times and an average of the obtained values was reported as ZOI (11).

Determination of the minimal inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) of each extract

MIC was carried out by the serial dilution method using 96-well microtiter plates. Eight dilutions of 80, 40, 20, 10, 5, 2.5, 1.25 and 0.62 mg/mL were prepared for the extracts with Mueller Hinton Broth medium. Also, 0.5 McFarland of inoculum (100 µl) was used. The microplates were incubated for 24 h at 37°C. The lowest concentrations without visible growth at the binocular microscope were recorded as MIC. The tubes with no visible turbidity was culture on MHA plate (50 µl) for determination of MBC and incubated for 18 h at 37 °C (12).

Bacterial adhesion to hydrocarbon (BATH) test

The BATH for each bacterial strain was assayed according to the following protocol: bacteria were grown overnight at 37ºC on MHA plate. Then, a loop of each bacteria was inoculated into a tube containing 9 ml of sterile phosphate buffered saline and vortexed. The OD_1 of this suspension was adjusted to 0.2-0.3 at 600 nm (OD_2) by spectrophotometer. Then, 200 µl of hexadecane was added to each tube and vortexed vigorously. The tubes were incubated at 30ºC for 20 minutes. Then, the secondary OD was read (OD_2). The BATH was calculated according to EQ1 formula

$$\text{EQ1: BATH (\%)} = \frac{OD1 - OD2}{OD1} \times 100 \quad (13)$$

Inhibition of biofilm formation

Biofilm formation in polystyrene microtiter plates was assayed as described by O'Toole and Kolter (14). Three different concentrations (12.5, 6.25 and 3.1 mg/ml) from each extract were pipetted (100 µl) into the wells of the microtiter plates. Then an overnight culture of each bacterial species was diluted 1:100 with fresh TSB and 100 µl of these inoculums were added to each well. Thereafter, microtiter plates were incubated for 24 h at 37°C. Three control wells were maintained for each test. These included wells containing the extract and growth medium (extract control), wells containing the growth medium and inoculum and wells containing only the growth medium.

The attached biofilm mass was quantified using crystal violet staining (15). After incubation, the media was aspirated and non-adherent cells were removed by washing the wells three times with sterile phosphate buffer saline (PBS). In order to fix the adherent cells, 150 µl of methanol 96% were added to each well for 15 min. The microtiter plates were then stained with 200 µl of crystal violet 1% (Merck, Germany) for 20 min. The plates were air dried and the CV bound to adherent cells was re-solubilized with 160 µl of 33% glacial acetic acid per well. The absorbance of each well was monitored with a microtiter plate reader (ELX-800, Biotec, India) at 630 nm. Percent inhibition of biofilm formation was calculated using EQ2 formula the ratio between the values of OD_{630nm} wells with and without the extracts.

$$\text{EQ2: Inhibition (\%)} = \frac{(\text{OD negative control} - \text{OD media control}) - (\text{OD test} - \text{OD extract control})}{\text{OD negative control} - \text{OD media control}} \times 100$$

Statistical analysis

Differences for individual parameters between control and treated groups were tested with Duncan's test by the analysis of variance (ANOVA) using SPSS Version 16.0 for Windows. Differences were considered significant if the P-value was less than 0.01, 0.05 and 0.001. All experiments were performed in triplicate and repeated three times.

Results

The inhibitory effects of the marine organism extracts against planktonic forms of six pathogenic bacteria

The ZOI of three extracts that was assayed by disc diffusion and agar-well plate methods are illustrated in Table 1. *C. lloydii* extract had antimicrobial activity against all six bacteria. *Callyspongia sp.* extract showed a better antibacterial activity in the agar-well plate test than the disc diffusion test. The MIC and MBC results are presented in Table 1. According to this table, the values of MIC and MBC are different for each bacterial strain. However, the 10-40 mg/ml concentration for MIC and 20-80 mg/ml for MBC were dominant between tested concentrations of three marine organism extracts.

Table 1. The antimicrobial effect of Callyspongia sp., Z. sansibaricus, and C. lloydii against six planktonic bacteria

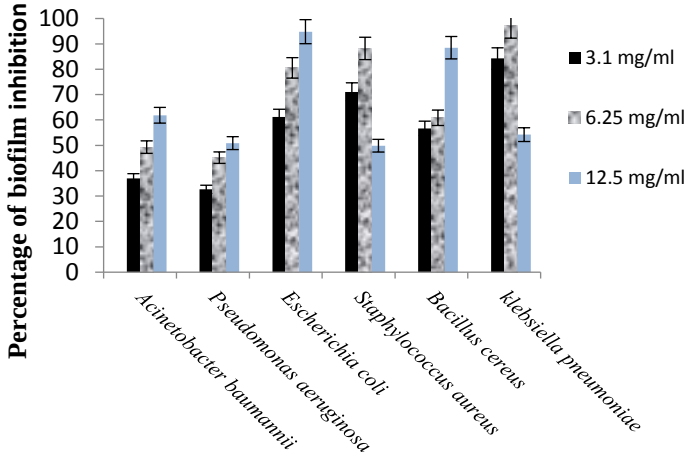
	extracts	*Pseudomonas aeruginosa*	*Acinetobacter baumannii*	*Bacillus cereus*	*Klebsiella pneumoniae*	*Escherichia coli*	*Staphylococcus aureus*
Disk diffusion (dia: mm±SD)	*Callyspongia sp.*	0	0	0	8±0.3	0	18±1.5
	Z. sansibaricus	8±0.3	10±0.2	0	0	8±0.6	10±0.8
	C. lloydii	8±0.4	10±0.7	8±0.4	20±0.7	8±0.5	10±0.7
Agar well plate (dia: mm±SD)	*Callyspongia sp.*	12±0.5	10±0.8	15±0.4	12±0.2	7±0.2	11±0.5
	Z. sansibaricus	16±0.2	10±0.3	13±0.2	10±0.8	10±0.5	11±0.3
	C. lloydii	12±0.8	18±0.5	20±0.8	0	15±0.3	20±0.2
MIC (mg/ml)	*Callyspongia sp.*	40	20	20	20	10	20
	Z. sansibaricus	10	10	10	10	10	20
	C. lloydii	20	40	10	10	20	40
MBC (mg/ml)	*Callyspongia sp.*	80	80	40	40	40	80
	Z. sansibaricus	20	40	40	20	40	80
	C. lloydii	80	80	40	20	80	80

The anti-biofilm activity of the marine organism extracts against pathogenic bacteria

The BATH results showed that all bacterial strains had the ability of biofilm formation. The BATH values for pathogenic bacteria were as follows: *P. aeruginosa* (29.31%), *A. baumannii* (68.09%), *B. cereus* (6.25%), *K. pneumoniae* (22.73%), *E. coli* (9.86%), *and S. aureus* (42.15%).

The effect of three concentration of extracts on pathogenic bacteria were studied. The results of extracts on the inhibition from biofilm formation are shown in Figure 2. *Callyspongia sp.*extract had the lowest inhibitory effect against biofilm formation of *P. aeruginosa* (32.65%) and the highest inhibitory effect against biofilm formation of *k. pneumoniae* (97.12%). From Figure2, it can be concluded that an increase in the concentration of sea anemone extracts escalates the inhibition of biofilm formation.

A)



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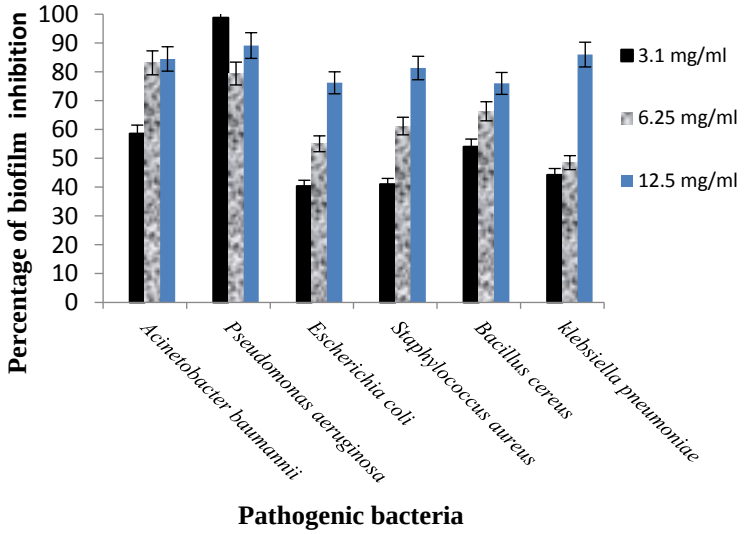

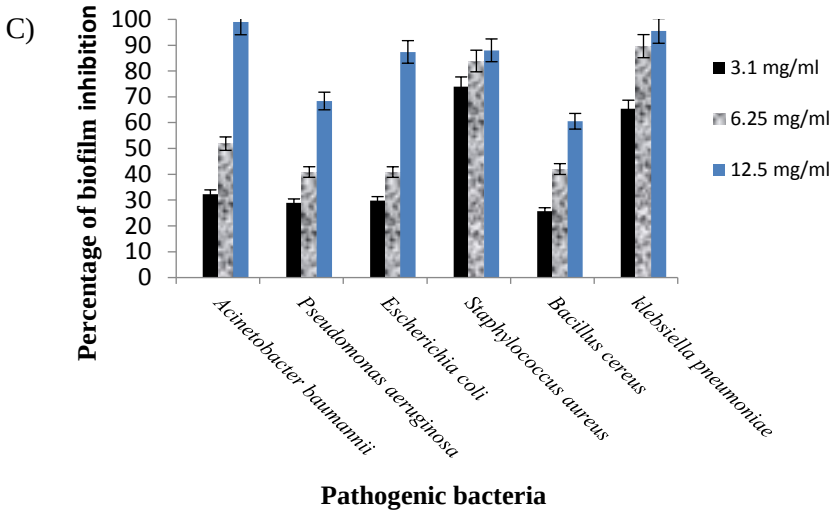

Figure 2. Percentage reduction of biofilm formation for test bacteria treated with different concentrations of the extracts for 24 hours: A) Sponge Callyspongia sp. B) Sea anemone Z. sansibaricus C) Sea anemone C. lloydii. In this research, a combination solvent dichloromethane: methanol (1:1 v/v) for the extraction of sponge and a methanol solvent for the extraction of sea anemones were used.

Statistical analysis

The effect of type of bacteria and different concentrations of three extracts on biofilm formation were analyzed statistically by Duncan's test. The results are presented in Table 2. Findings confirmed that both of the sea anemone extracts were statistically effective for biofilm inhibition.

Table 2. Statistical analysis of the results of bacterial biofilm formation by Duncan's test

		Biofilm formation		
Extracts	**Variables**	**Df**	**Ms**	**Sig**
Callyspongia sp.	Type of Bacteria	5	0.097	**
	Concentration of extract (mg/ml)	2	0.041	_
	total	7		
Z. sansibaricus	Type of Bacteria	5	0.044	*
	Concentration of extract (mg/ml)	2	0.105	**
	total	7		
C. lloydii	Type of Bacteria	5	0.088	**
	Concentration of extract (mg/ml)	2	0.232	***
	total	7		

Ms; Mean square, Df: Degrees of freedom, Sig: Significant.

*: p< 0/05, **: p< 0/01, ***: p< 0/001, -: No significant level.

Discussion

The detection of antibiotics and the consequent development of synthetic antimicrobial compounds have changed our therapeutic approach towards infectious diseases and enhanced the quality and length of life for humans and other organisms (7).

Secondary metabolites from aquatic organisms have shown to be an exceptionally valuable source of small molecules with pharmacological activities potentially advantageous to human health (2).

Govinden *et al* examined the antimicrobial activity of crude extract of *Biemna tubulosa* and *Stylissa sp.* against *E. coli, S. aureus* and *Enterococcus faecalis.* Their chemical screening showed that the major composition was tannins, saponins, terpenes, alkaloids, and phenols (16). However, Darah *et al*. reported that a methanolic extract of *Haliclona sp*. was not effective against *E.coli* (17).

The antimicrobial effect of sponge extract (methanol-acetone) obtained from Mannar Bay region in the southeast of India shows that many sponges tested such as *Haliclona cribricutis, Spongia officinalis, Myrmekioderma granulata, Oceanapia fistulosa,* and *Fasciospongia cavernosa* cannot prevent growth of *P. aeruginosa*. Also, the extract of *Haliclona exigua, Petrosia nigricans* and *Dysidea spp.* in 500 µg/ml concentration prevents the growth of pathogenic bacteria (18).

In this study, the polar and non-polar extracts of *Callyspongia sp*. sponge had a well antimicrobial effect on both single and biofilm forms of all six studied bacterial strains. This finding was confirmed by the statistical analysis. Among the studied bacteria, the least effect of the extract was observed on the control of biofilm of *P. aeruginosa*. Differences in the results of this study with other similar studies are likely to be affected by factors such as the species of marine sponge and the type of solvent used for extraction. It is very important that different ecological conditions of sponges can lead to differences in harmful microorganisms and their symbiosis, and ultimately produce different bioactive compounds (19).Some symbiotic microbes such as sponge-associated actinomycetes can create bioactive compounds in parallel. It is estimated that 22 % of compounds received from marine sponge related to symbiosis with actinomycetes (19).

Between the secondary metabolites produced by aquatic sponges, a vast diversity of steroids, isoprenoids, non-isoprenoids, quinones, nitrogen and nitrogen-sulfur heterocyclic compounds, alkaloids, peptides, and terpenes are included. It is important to mention that some of these compounds divided from marine sponges are only synthesized in symbiotic relationships with fungi, microalgae, archea, cyanobacteria, and bacteria (20, 21).

Newbold *et al.* collected 33 sponges from the Caribbean Sea in 1999 and evaluated the antibacterial activity of these sponges. They concluded that 48% of collected sponges had antibacterial activity (22).

During the early 1990s, the activity of polar and non-polar extracts from soft corals (Anthozoa: Octocorallia) *Plexaura homomalla, Pseudoplexaura flagellosa, Plexaurella fusifera, Eunicea clavigera, Eunicea tourneforti, Eunicea laciniata, Eunicea*

calyculata (Plexauridae), and *Pseudopterogorgia americana* (Gorgonidae) were tested on marine bacteria and on human pathogens (*Vibrio harveyi, P. aeruginosa, S. marcescens, S. aureus, B. megaterium* and *E. coli*). The inhibition of microbes seeded in agar plates was evaluated after the application of 6.2 mm paper disks soaked in cnidarian extracts and incubated overnight at 37℃. to obtain the ultimate result. *P. homomalla* and *P. flagellosa* extracts exhibited the main antimicrobial activity with inhibition scores of 18.3 and 15.0, respectively (23).

During the last decade, primary reports on the antimicrobial activity of crude aqueous methanol extracts from *Leptogorgia virgulata* were published. The extracts inhibited the growth of *Vibrio harveyii* and *Micrococcus luteus* (extraction of >0.5 g of tissue), and of *E. coli* (extraction of 2.0 g of tissue) (24).

Consequently, Tadesse *et al*. examined the extracts from *Alcyonum digitatum* on Gram-negative and Gram-positive bacteria. *E. coli* and the fish pathogen *Listonella anguillarum* serotype O2 (Gram-negatives) exhibited resistance, while the Gram-positives *S. aureus* and *Corynebacterium glutamicum* were sensitive (MICs: 80 mg/mL) (25).

Thermo-stable proteases and antimicrobial peptides have been purified from the body and tentacles of the sea anemones *Actinia equina* and *Anemonia sulcata*. These compounds had antifungal activity. The mucus of *A. equina* has been newly shown as a source of antimicrobial lysozyme-like compounds, indicating lysozyme-like activity and thereby possessing activity as an antifouling agent, countering the settlement of bacteria, as the primary colonizers in marine waters. The activity of the mucus was observed to be dependent on pH (highest diameter of lysis was observed at pH 6.0), ionic strength, and temperature. Lysis was reported to increase after dialysis of mucus at pH 6.0, 0.175 ionic strength, and 37 ºC temperature resulting in a diameter of lysis of 16.2 ± 0.5 mm, corresponding to 2.21 mg/mL of hen egg-white lysozyme. Considering that seawater has a higher pH and ionic force, the activity of this mucus against marine bacteria remains to be explained. The activity against *Micrococcus lysodeikticus* identified to be beneficial for the assay of lysozyme, and the satisfactory results achieved at 37 ºC make *A. equina* mucus an interesting prospect for future extensions in fighting pathogenic microbes (7).

John *et al*. in 2015 evaluated the inhibitory effect of *Stichodactyla haddoni* and *Anthopleura elegantissima* extracts against human pathogens. The diethyl ether crud extracts of these sea anemone species exhibited good activity against gram positive and gram negative bacteria. A few studies was done in the field of anti-biofilm activity of bioactive compounds from marine animals (26, 27). Bragadeeswaran *et al* searched the antifouling activity of two sea anemone extracts (*Heteractis aurora* and *H. magnifica*) against seven bacterial biofilms. They concluded that the crude extract of *H. magnifica* had the highest inhibition zone (18 mm) against *Pseudomonas sp*. and *E. coli.* The minimum inhibition zone (3 mm) was observed against *P. aeruginosa*, *Micrococcus sp*., and *B. cereus* for methanol, acetone, and dichloromethane extracts, respectively (28, 29, 30).

According to table 1 and Figure2, both methanol extracts of sea anemone *Z. sansibaricus* and *C. lloydii* in this study had an appropriate antimicrobial activity against all 6 pathogenic bacteria. We assume that methanol solvent is suitable for extraction. In most experiments in this study, there was a direct link between increased concentrations of sea anemone extract and a bacterial biofilm inhibitory activity. We used the complete form of sea anemone for extraction. For this reason, it cannot be exactly determined that the observed antimicrobial activity relates to the toxins secreted by the sea anemone or to the symbiotic microorganisms, or both.

Factors such as the problem in collecting specimens, the scarcity and quantity of active extracts and the broad heterogeneity of compounds are among the challenges encountered in order to develop novel therapeutic agents from marine organisms (7).

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