

MARINE INVERTEBRATES FROM COLOMBIAN CARIBBEAN COAST: A POTENTIAL SOURCE OF BIOACTIVE MOLECULES

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Abstract

Marine invertebrates and particularly their extracts are widely studied because these species represent a potential biological source of new molecules with biological activity. This study presents the antibacterial and cytotoxic effect of crude extracts from five different marine invertebrate species (*Eunicea sp.*, *Suberites sp.*, *Ophiocoma sp.*, *Syllis sp.* and *Lytechinus sp.*). It was found that crude extracts of octocoral *Eunicea sp.* represent a potential source of molecules with antibacterial activity against clinical importance microorganisms as *K. pneumoniae* and against insect pest as shown by the insect cells bioactivity test. The resulting differences found between the results from this research with those reported by other authors, require deeper studies in order to establish the true origin of these bioactive molecules. New studies must consider that a close relationship of marine invertebrates with microflora has been reported meaning a potential role of microflora as producer of bioactive molecules.

Key words: Antimicrobials, Bioprospecting, Cytotoxic, Marine animals, Sf9

Introduction

Colombian marine biomes are considered threatened “biodiversity hotspots”, which should be studied because they are considered a natural source of molecules with a great potential to be used in different biotechnological approaches [1]. It is well known that marine invertebrates produce a great diversity of antimicrobial peptides (AMPs), which enables them to interact with microbes in their environment due to the positive charge and electrostatic forces of these molecules. It has been showed that AMPs can penetrate into the bacterial phospholipid bilayer by establishing amphiphilic structures within hydrophobic environments [2]. Microorganisms such as Gram-negative and Gram-positive bacteria, fungi as well as yeast could become a serious medical problem, mainly due to the emergence of drug-resistant strains [3]. These microorganisms are responsible for nosocomial infections and they have created a recurring problem around antibiotic-based therapies, especially in the absence of new compounds that could be used for control of this type of infectious agents. The first penicillin-resistant Pneumococci appeared in 1967, since then numerous resistant microorganisms strain have appeared to date [4]. Currently the use of compounds derived from organisms, have taken a boom due to their natural properties, providing different modes of action that are more effective than synthetic molecules, and avoiding the development of resistance [3].

In addition, it has been showed that this kind of natural compound is also safer for the environment because they are biodegradable under specific conditions.

These compounds include metabolites from marine animals with potential to be used in different areas such as medicine, agroindustry, cosmetics, food among others. This research job represents at glance a systematic purpose of bioprospecting from marine animals by using antimicrobial and cytotoxic bioassays as initial screening model.

Methods

Biological material

The marine animals were collected through scuba diving in rocky shores of the Colombian Caribbean, located in the town Taganga (Santa Marta – Magdalena, 11°16'08.89"N, 74°11'50.21"O). All samples were transported at 4 °C to the laboratory of microbial biotechnology at the Universidad del Atlántico (205B); cut into small pieces, washed thoroughly with distilled water and frozen at -20 °C for further analyses.

Antimicrobial activity

In order to evaluate the antimicrobial activity from several Colombian Caribbean marine invertebrates, aqueous extracts were prepared triturating each animal total body with 15 mL of Tris-HCl buffer. This extract was centrifuged at 5000 x g at 4 °C, and the resulting supernatant was used in the bacterial inhibition test. The inhibitory effect from the extract was carried out against opportunistic pathogens as *Escherichia* (ATCC 8739), *Salmonella* (ATCC 13311), *Klebsiella* (ATCC 13883), *Proteus* (ATCC 31008), *Staphylococcus* (ATCC 25923) and *Pseudomonas* (ATCC 27853), in 96 well microtiter and the final volume in each well was adjusted to 100 µL. Each treatment consisted of 90 µL LB culture medium (50% Bactotryptone, 25% yeast extract, 35% NaCl, diluted in distilled water), 95 µL of each extract and 5 µL of respective overnight bacterial culture, using a negative control (distilled water) and positive control (chloramphenicol 40µg.ml⁻¹). Bacterial cultures were incubating at 37 °C and their growth was monitored at OD 600 until reaching the stationary phase. Each treatment was carried out by triplicate. The percentage of inhibition was calculated from log phase to each microorganism using the following equation[5].

$$\%Inh = \frac{OD_{NC} - OD_T}{OD_{NC}} * 100$$

OD_{NC}= Absorbance of negative control

OD_T= Absorbance of treatment

Cell Viability Tests using Insect Cell Lines

The bioassays were performed using insect cell line Sf9 from *Spodoptera frugiperda*, that was obtained from the envelope of the ovary pupa [6]. Insect cells were maintained at 27 °C in TNM-FH medium supplemented with 10% (v/v) FCS, and 1% penicillin-streptomycin-amphotericin. Additionally, the cultures were split every 5 days for maintenance. The insect cells selected for experiment were seeded in microtiter six well plates at the early stationary phase with a starting density of 2 X 10⁵ cells.ml⁻¹. Then, 10 µL of aqueous extracts obtained from marine animals were added to each well and cells left without treatment (water) were used as negative control.

Plates were incubated at 27 °C and analyzed at 72 h of exposure. Finally, cells were homogenized, added to the solution of 0.4% trypan blue dye (1:1, v/v) and observed in a Neubauer chamber using light microscopy (20X), following the methodology described by Valencia

et al (2011) [14]. To perform cell counting, cells blue stained were considered no viable and cells clear as viable.

Statistical analysis

For statistical analysis, all tests were evaluated in triplicate. Data were compared by one-way analysis of variance followed by Tukey's test when significant differences were found at $p < 0.05$. The cell viability percentage was calculated and corrected by the negative control (Schneider-Orelli formula). All descriptive analysis, normal distribution, homogeneity of variance, difference between treatments and plots were performed using SPSS (version 17.0) for Windows (SPSS, Inc., Chicago, IL).

Results

Antibacterial effects

The Figures 1 (A to F), show growth curves by organism and treatment. These results suggest that both *P. aeruginosa* and *P. Mirabilis* strains, display resistance to the antibiotic chloramphenicol. Likewise, the growth curves indicate that only the extract from Coral *Eunicea* sp. specie was efficient to inhibit growth of both Gram (+) and Gram (-) bacteria so, the extract from this specie was selected to perform antibacterial bioassays.

Inhibition percentages are presented at the Table 1. It was calculated by using the absorbance values obtained at four hours after starting the cell culture because, at this time all strains were in exponential growth phase. It was found that the Coral *Eunicea* sp. extract is a potent growth inhibitor of *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* strains; while in the case of *Salmonella thiphymurium* and *Pseudomonas aeruginosa*, the extract only reaches an inhibition percentage of 50% and 12% respectively. Besides, *Proteus mirabilis* was totally resistant to the potential negative effect of this extract.

Cytotoxic effects on insect cell line

Trypan blue dye (0.4%) was added to insect cell cultures in a ratio of 1:1 and observed by light microscopy. All extracts from marine animals collected from Colombian Caribbean Rocky Shores showed effect on *Spodoptera frugiperda* insect cell line Sf9; decreasing cell viability up to 96% when compared to control treatment (Figure 2). However, only samples from *Eunicea* sp. and *Syllis* sp. show statistically significant difference in comparison with the negative control ($p < 0.05$). In addition, structural changes and insect intoxication

symptoms were observed under 40X and 100X light microscopy after 72 h of exposure (Figure 3). Observed changes included vacuolization, mitochondria swelled with aspect of spherical vacuoles, cell membrane deformation, generalized stress and cellular lyses .

Discussion

The growing number of infectious diseases caused by bacteria, fungi and viruses, coupled with the emergence of resistant microorganisms to chemotherapeutic agents; constitutes the reason why numerous studies have focused on the search for new sources of bioactive molecules whose mechanisms of action are in constant change in order to evade metabolic pathways commonly used by these resistant organisms[5]. Plants, animals, bacteria and fungi have been extensively studied in the search for new molecules with this specific biological activity [4]; but are the marine invertebrates the organism that currently occupy the interest of many researchers worldwide, who have reported a wide variety of molecules with anti-inflammatory, anti-cancer, antiviral, antifungal and antibacterial activities [1,7–9].

In this study, we report potent antibacterial activity from aqueous extracts of octocoral *Eunicea* sp. Some previous and related studies corroborate the findings that are showed in this report [10]. However, other research describe antibiofilm activity but not antibacterial activity from crude extracts of this kind of octocoral [1]. These reported differences may be related to the true source of bioactive molecules as some studies report that the isolated molecules of these extracts can be produced by the microflora associated to the organism but not properly coming from the invertebrate cells [3,11]. These findings may explain the results obtained in this study where antibacterial activity of the crude extracts from the species reported *Suberites* sp., *Ophiocoma* sp., *Syllis* sp. and *Lytechinus* sp. was not observed; while several other studies report antibacterial activity from these extracts. This suggests that aspects such as: the diversity of microflora associated with these invertebrates, climatic and collection sites have influence on the biological activity and potential of these compounds. It could be recommended if a systematic search for new molecules is bioguided, and then correlated with biological activity and environmental conditions.

Compounds from marine organisms may offer an alternative to drugs [12] or to chemical insecticides [13]. This is the first cytotoxic activity report in which extracts from marine animal were

assayed on a *Spodoptera frugiperda* cell line (Sf9). In spite of the fact that cell viability did not decrease significantly, with percentages above 80% (Figure 2); result important to point out that these experiments were conducted with crude extracts at a concentration lower than 10 ppm, constituting the first step of a bioprospecting way. Although, the molecules explored exhibited insecticidal action, further research is needed to evaluate its real potential to be considered for insect pest control in the near future [14]. Studies evaluating body fluids, extracts or compounds from marine organisms have been carried out on human cell lines in a search for anticancer agents, drug candidates with potential to be used at clinical level in the future [15], spermicides, antiphytoplankton compounds, active agents on human colon carcinoma cells [16] or molecules with antioxidant activity [17]. Many studies with main focus on the identification of bioactive peptides from marine sources describes isolation procedures and their pharmacological properties [18]. In this current approach, we presented results of biological activity from different marine animal extracts such as *Eunicea* sp. and *Syllis* sp.; which showed high values of biological activity on the insect cell line Sf 9 (Figure 2), and cytotoxic effects on structural aspects of the cells Sf 9 (Figure 3). These results demonstrates that sea animals are not only a potential source to find useful compounds as an alternative to conventional antibiotics [7] but also a biological source in order to select metabolites acting as insecticidal agents as described by El Sayed et al., (1997) [19]. Furthermore, trypan blue methodology used in this report represent a rapid, efficient and economical strategy to select extracts from marine animals that could contain metabolites with potential to be used as an alternative to insecticides.

This study is therefore relevant, because the increasing incidence of resistant pathogenic bacteria to conventional antibiotics. In addition, is important to mention that therapeutic resources available for the treatment of many hospital-acquired infections are depleted because of the increasing inefficiency of antibiotics, thus resulting in the need to find new antimicrobial agents, especially if we consider that many bacteria have developed resistance to conventional agents [9]. Likewise, the increasing use of pesticides in agriculture to control insect pests during the last decades has led to problems of environmental pollution and health risks to farmers. For this reason, the bioprospecting of new molecules from diverse biological sources may be an alternative

solution to seek natural products with insecticidal effects and to replace chemical pesticides. Nevertheless, further characterizations is needed in order to design natural products based in these compounds as new biotechnological solution.

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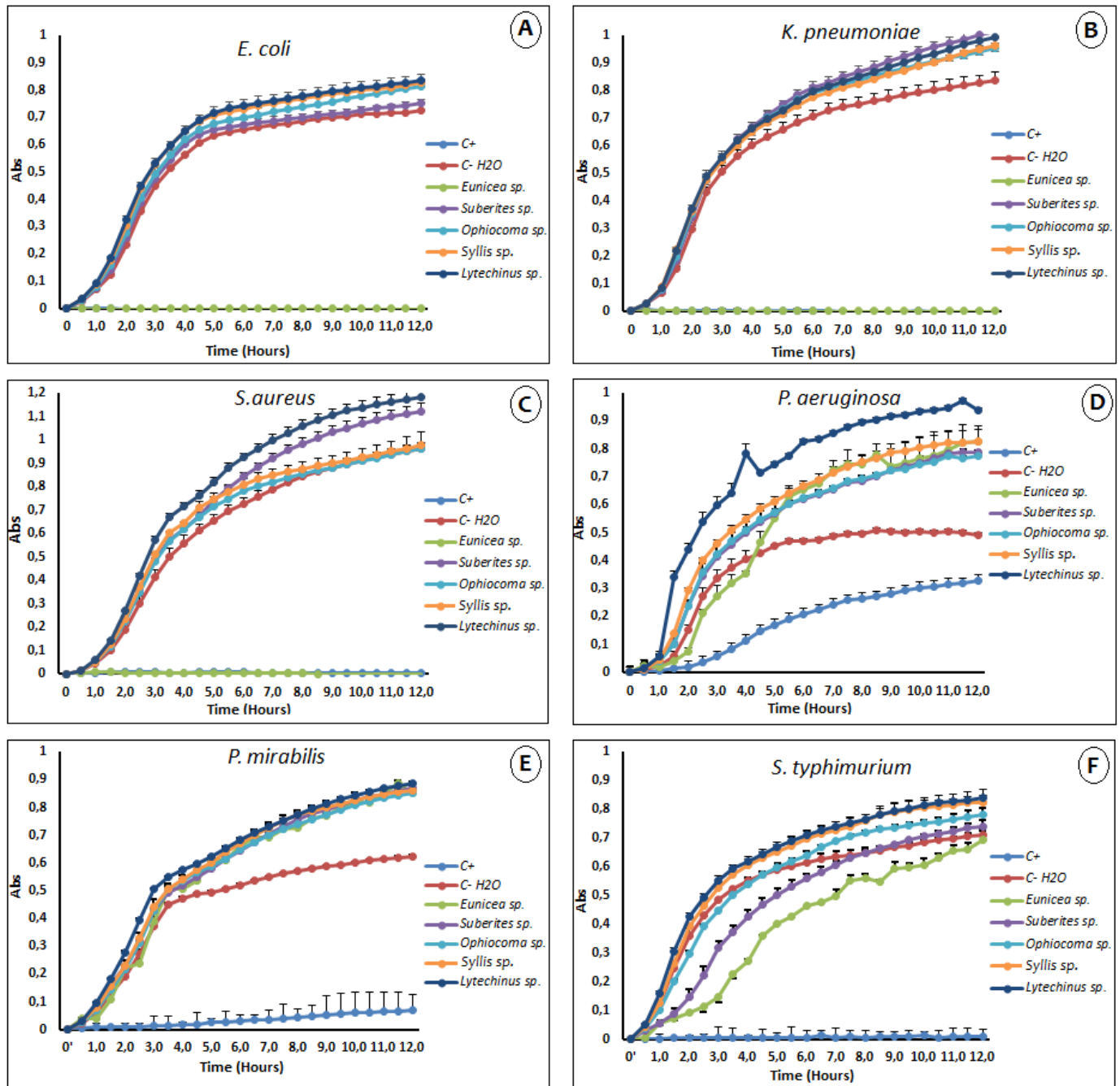


Figure 1. Growth curve of different bacteria tested against marine invertebrate extracts. Data are expressed as mean \pm standard deviation from three replicates.

Table 1. Inhibition percentages obtained by each extract against all strains that were tested. Data are expressed as mean from three replicates.

Bacteria/ Sample	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>P. mirabilis</i>	<i>S. typhimurium</i>
<i>Eunicea sp.</i>	100,0	100,3	99,0	12,2	-7,1	50,5
<i>Suberites sp.</i>	-6,6	-10,7	-11,1	-23,8	-9,8	22,5
<i>Ophiocoma sp.</i>	-9,8	-9,5	-11,4	-26,0	-13,7	2,1
<i>Syllis sp.</i>	-15,0	-7,6	-16,0	-36,1	-14,6	-9,6
<i>Lytechinus sp.</i>	-15,7	-9,6	-28,7	-142,2	-21,7	-12,5

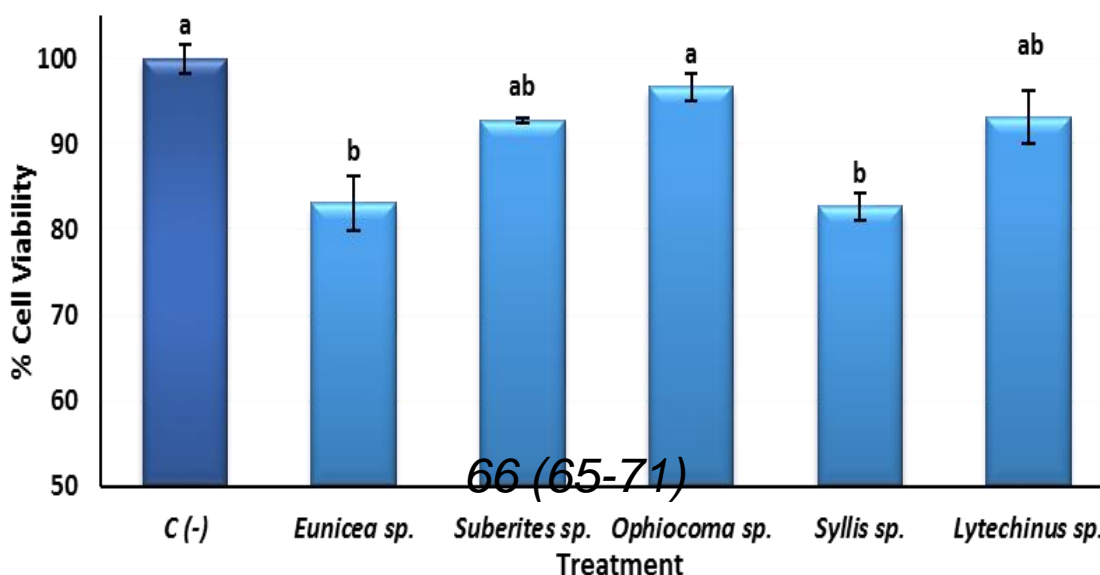


Figure 2. Results of Sf9 cell viability test against crude extracts from marine invertebrates. Data are expressed as mean ± standard deviation from three replicates. Significant difference between treatments are indicated by different letters Tukey ($p < 0.05$).

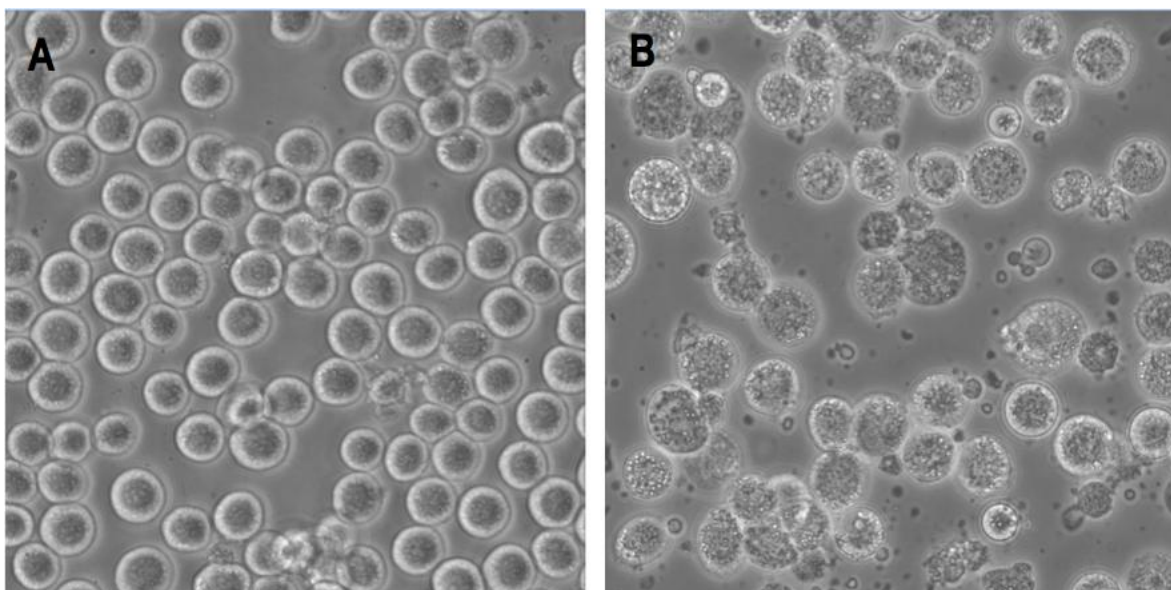


Figure 3. Analysis of the in vitro effects and images of light microscopy of Sf9 ovary cells treated with extracts from marine animals after 72 h of exposure (40X). A. Negative control Sf9 cells. B. Cytotoxic activity of *Eunicea sp.* crude extract (10 μ L) on Sf9 cells.