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Extraction and Screening of Bioactive Compounds with Antimicrobial Properties from Selected Species of Mollusk and Crustacean

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Abstract

The resident animals of polluted areas possess possibly novel antimicrobials towards off infections. Selected species of sea invertebrates *Perna viridis (Bivalve), Nerita albicilla (Gastropoda)* and *Ozius rugulosus (Crustacean)* were tested for antibacterial activity against human pathogens *Acinetobacter baumannii, Pseudomonas aeruginosa and Escherichia coli K1*. The best results were obtained by methanol extracts of selected sea animals against *Pseudomonas aeruginosa*. The overall aim of this study is to gather preliminary information about the antimicrobial activity of invertebrates from Manora channel for potential use in the development of new antibiotics.

Keywords: Antimicrobials; Antibacterial activity; Bactericidal activity; Mannora Channel

Introduction

The marine life constitute almost 80% of the world biota [1] and are source of unique natural products used as food, fragrances, pigments, insecticides, medicines etc. More or less 10,000 pharmacologically bioactive compounds have been derived from marine invertebrates such as tunicates, sponges, soft corals, sea hares, nudibranchs, bryozoans, sea slugs and other marine organisms [2].

The secondary metabolites derived from number of marine animals possess antibiotic, anti-parasitic, antiviral and anti-cancer activities [3,4]. The Tyrian purple an ancient dye pigment is the first natural product of marine origin reported in literature. Many mollusks mantle cavity produces mucus e.g. Muricid gastropods (rock snail) which defend the developing larvae against microbial infection [5].

Besides that wide range of bioactive metabolites known to occur in sponge genera Helicona, Petrosia and Discodema that act as powerful anti-cancer and anti-inflammatory agents [6]. Marine toxins such as tetrodoxin, soxitoxins, ciguatoxins and brachtoxin serve as specific sodium channel blockers and are useful in neurophysiological and neuropharmacological studies [7-10].

Among marine invertebrates, marine molluscs are the good source of bioactive metabolites. The bioactive compounds extracted from many classes of molluscs exhibit antitumor, antileukemic, antibacterial and antiviral properties [11,12].

Marine invertebrates rely solely on innate immune mechanisms that include both humoral and cellular responses. Humoral immunity in marine invertebrates is characterized by antimicrobial agents present in the blood cells and plasma [13]. Cellular immunity in marine invertebrates is based on cell defense reactions, including encapsulation, nodule formation, and phagocytosis [13].

The cellular component of marine invertebrate immunity is mediated by hemocytes (blood cell) of, motile cells that phagocytize microbes and secrete soluble antimicrobial and cytotoxic substances into the hemolymph (circulatory fluid of invertebrates comparable to the blood) [14].

Protein is a major biochemical constituent in all invertebrate and received highly attention due to their potential bioactive and functional properties. However, interest in marine proteins might not be only directly correlated to the intact protein, but also to the possibility of generating bioactive peptides. In this sense, different peptides derived from marine proteins have been identified as having antimicrobial activities [15].

The recent development in research on multi-drug-resistant bacteria suggests that animals living in unsanitary and unhygienic conditions have developed ways of protecting themselves against pathogenic microorganisms [16-22].

Despite being the hot spot of pollution, the Manora Channel is a rich source of invertebrate and vertebrate fauna [18,23-26]. In this present study Antibacterial activity was investigated against human pathogens *A. baumannii, P. aeruginosa* and *E. coli* k1 to search for new bioactive compounds. Water and Methanol extracts of *Perna viridis, Nerita albicilla* and *Ozius rugulosus* were tested for bactericidal effect.

Materials and Methods

Sampling site

Manora Channel lies in south of the city of Karachi at 24°47”N, 66°58”E. It is roughly 8 km long and 850 to 1000 m wide. The Channel is a hot spot of pollution. It is heavily polluted by untreated industrial wastewater and metropolitan municipal sewage discharged into coastal waters through Layari and Malir rivers. The Channel also receives all sorts of pollutants from Karachi Fish Harbour, shipyards, power plants and ships harbouring at Karachi Port. Despite being polluted, the channel is a rich source of invertebrate and vertebrate fauna (is all of the animal life of any particular region or time).

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Animals selected for tissue extraction

The specimens of *Nerita albicilla* (gastropod), *Perna viridis* (mussel) and brachyuran crabs *Ozius rugulosus*, were brought live to the laboratory. The animals were dissected to separate their gills, gut and gonad. Tissue extracts were used for antibacterial assay.

Taxonomy and description of animals used for extraction

Phyum: Mollusca

Class: Gastropoda

Family: Neritidae

Genus: *Nerita*

Species: *N. albicilla* Linnaeus, 1758

Description: Nerita albicilla, is a species of sea snail, a marine gastropod. It is found on rocky cliffs, on rocks in the littoral fringe, and sometimes on mangrove trees [27] (Figures 2A and 2B).

Class: Bivalvia

Order: Lamellibranchia

Family: Mytilidae

Genus: *Perna*

Species: *P. viridis* Linnaeus, 1758

Description: The Asian Green Mussel, *Perna viridis*, is an economically noteworthy mussel, a bivalve in the family Mytilidae. The native range of the Asian green mussel broadly encompasses the Asia-Pacific and Indo-Pacific regions [28] (Figures 2C-2E).

Phylum: Arthropoda

Class: Crustacea

Family: Eriphiidae

Genus: *Ozius*

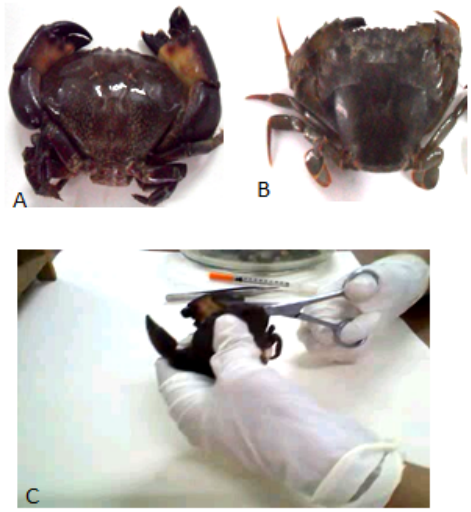

Figure 1: (A and B) Ozius rugulosus. (C) Turned the crab so its dorsal side is up. Inserted the tip of a heavy scissor beneath the lateral, posterior edge of the carapace (A bony or chitinous case or covering the back part of animal), and made a cut around the periphery of the carapace on its dorsal surface.

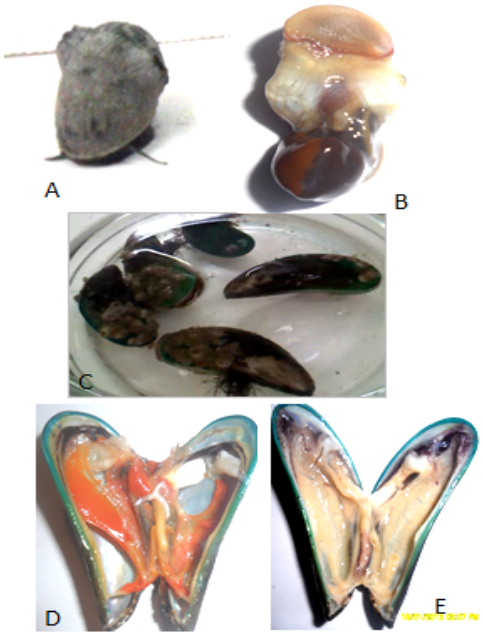

Figure 2: (A) Shell, Nerita albicilla **(B)** Dissected Nerita albicilla. Dorsal view of animal after shell and mantle (a fold of body wall) removed. **(C)** Live specimen Perna viridis kept in aquarium. **(D and E)** Perna viridis was differentiated into male (white) and female (Red to Orange) on the basis of gonadal tissue color.

Species: *O. rugulosus* Stimpson, 1858

Description: *Ozius rugulosus* is a genus of crabs in the family Menippidae. This was found under a rock just at the edge of the beach rock. Distribution: Indo-west Pacific Oceans and Arabian Sea. Ecology: Rocky shore inhabits the crevices (A narrow opening resulting from crack of rocks) of rocks [29] (Figure 1).

Test microorganisms

Test microorganisms *Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli K1* were obtained from microbial culture collection (MDL Lab.), Aga Khan University and Hospital, Karachi Pakistan.

Extraction of Bioactive compounds

After collection, the samples were rinsed with sterile seawater to remove associated debris. The bioactive compounds (Antibacterial peptides) were extracted in distilled water and methanol. Prepared sample as 10 gm of tissue+5 ml distilled water or 10 gm tissue+5 ml methanol. Tissues (gills, gut, gonad) were homogenized with the help of tissue homogenizer (Tekmar Tissuemizer, Type SDT 18/10) at 110 V. Later on tissues were sonicated by (Misonix Q55 Sonicator, (Ultrasonic Liquid Processor) used to speed dissolution, by breaking intermolecular interactions to disrupt cell membranes and release cellular contents (for three minutes). Then samples were centrifuged at 24696 g for 45 minutes and the supernatant was collected and stored at -80°C.

Antibacterial peptides

Antimicrobial peptides are a major component of the innate immune defense system in marine invertebrates. They are defined as molecules less than 10 kDa in mass which show antimicrobial properties [30] and provide an immediate and rapid response to invading microorganisms

[14]. There is evidence that antimicrobial peptides are widespread in invertebrates [31], especially in tissues such as gut and respiratory organs in marine invertebrates.

Antibacterial assay

Pathogenic bacterial strains (*A. baumannii, P. aeruginosa and E. coli K1*) were inoculated in sterile nutrient broth (Lauria broth) and incubated at 37°C for 24 hours. Various concentration of tissue extract with water and methanol were incubated with 10×6 bacterial cells and final volume was adjusted to 200 µl. Methanol alone was used as negative control. Then the tubes were incubated for 2 hours at 37°C and bacterial counts were enumerated by serial dilution and plating on nutrient agar plates as described by Khan et al. To determine the number of colony-forming units (cfu), plates were incubated overnight at 37°C. Bactericidal effect (BE) was determined as the percentage of bacteria surviving relative to the control (BE=100-(cfu in crude tissue extract/cfu in control×100). The entire test was carried out in triplicates.

Results

The methanol extracts and water extracts of marine molluscs, gastropods and crustaceans tested against 3 biofilm bacteria which showed bactericidal activity (Table 1). The overall screening showed highest activity in gut of *Perna viridis* 99.63% against *P. aeruginosa.* Low activity was observed in gonad of Perna viridis against *E. coli K1*. Other Methanol extracts were also able to produce bacterial inhibition against *E. coli K1, A. baumannii* and *P. aeruginosa*. Methanol extracts had better antibacterial effects rather than water extracts. Methanol alone was used as negative control. The extracts having antibacterial activity was determined by tube dilution assay and results were summarized in (Table 1 and Figures 3-5).

Discussion

Seawater is highly susceptible to contamination due to nearby municipal waste water. The more serious the contamination, the more abundant the distribution of the pathogens.

Invertebrate	Bactericidal Activity %		
Perna viridis			
	A. baumannii	*E.coli k1*	*P. aeruginosa*
Gills	82.4	47.36	99.4
GIT	94.4	34.21	99.6
Gonad	92.22	21.05	95.61
Nerita albicilla			
	A. baumannii	*E.coli k1*	*P. aeruginosa*
Gills	62.84	34.21	99.12
GIT	77.5	74.3	97.5
Gonad	28.17	84.8	90
Ozius rugulosus			
	A. baumannii	*E.coli k1*	*P. aeruginosa*
Gills	93.8	98.4	98.1
GIT	75	89.8	96.7
Gonad	85.2	82.8	88

Note: This table indicated *P. aeruginosa* maximally inhibited by methanol extracts of *Perna viridis, Nerita albicilla* and *Ozius rugulosus*. Methanol extracts of marine invertebrates were also capable to inhibit *A. baumannii and E. coli k1* growth greatly. Low antibacterial activity was seen in case of Perna viridis (gonads) with minimal inhibition at 21.0% against *E. coli K1*. Marine invertebrates' extracts had the overall best activity against pathogens.

Table 1: Effect of (methanol) tissue extracts on survival of *A. baumannii, P. aeruginosa* and *E. coli K1*. **B.E:** The effectiveness and ability of an antimicrobial agent to inhibit and kill bacteria.

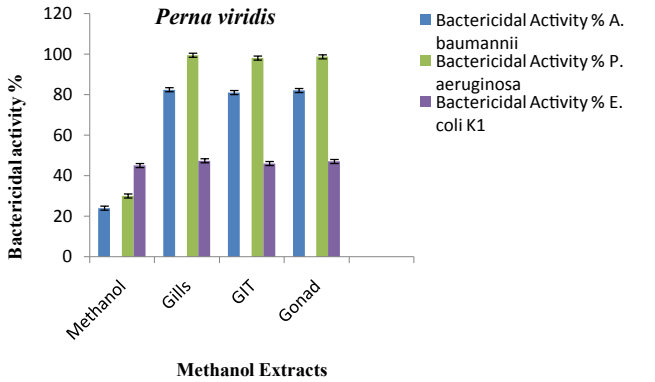

Figure 3: Bactericidal activity. *P. aeruginosa*, *A. baumannii* and *E. coli* k1 were inhibited by *Perna viridis* (methanol extracts). GIT tissues had the overall best activity for all three selected bacteria. Methanol solvent (negative control) showed very low activity against all 3 selected bacteria.

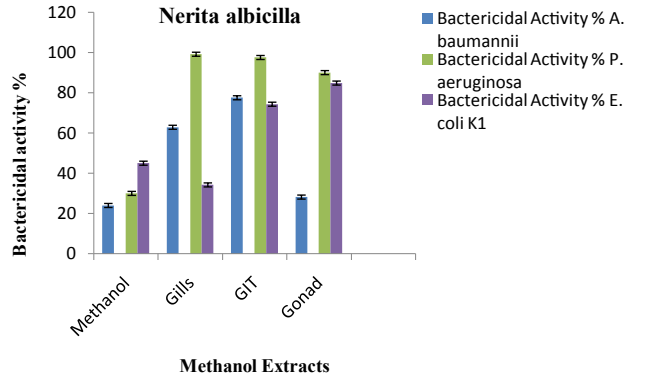

Figure 4: Bactericidal activity. *P. aeruginosa, A. baumannii* and *E.coli k1* were inhibited by *Nerita albicilla* (methanol extracts). GIT tissues had the overall best activity but less than *Perna viridis* gut extracts for all three selected bacteria. Methanol solvent (negative control) showed very low activity against all 3 selected bacteria.

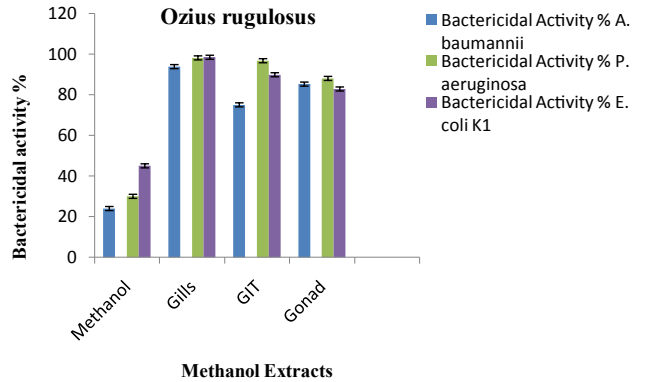

Figure 5: Bactericidal activity. *P. aeruginosa, A. baumannii* and *E. coli k1* were inhibited by *Ozius rugulosus* (Metnanol gills extracts). Gills tissues had the overall best activity for all three selected bacteria and selected gut tissues (*Perna viridis* and *Nerita albicilla*). Methanol solvent (negative control) showed very low activity against all 3 selected bacteria.

Animals living in filthy environment have developed ways of protecting themselves against pathogenic microorganisms [24].

More than hundred new antimicrobial compounds were isolated every year from marine invertebrates, such as sponges, gastropods, bivalves which shows broad spectrum of antimicrobial properties [32-34]. In recent years significant works have done antibacterial activities of mollusk. More than thousand new compounds has been categorized from marine invertebrates such as peptides, terpenes, polypropionates, nitrogenous compounds, polypeptides, macrolides, prostaglandins and fatty acid products, sterols and diverse compounds [34].

Both animals and plants possess potent, broad-spectrum antimicrobial peptides, which they use to fend off a wide range of microbes, including bacteria, fungi, viruses and protozoa [35].

Antimicrobial peptide defense in Crustacea has long been suspected. The isolation of several peptide factors displaying antimicrobial activities from crabs and shrimp. Prominent among crustacean antimicrobial peptides are the penaeidins, which display antifungal and antibacterial properties and were isolated from the hemolymph of the shrimp *Penaeus vannamei* [36].

The hemocytes of the shore crab *Carcinus maenas* have been shown to contain broad-spectrum antibacterial activity, and similar activity is displayed by the hemocytes of several other crustacean species [31,37].

Mollusks rely predominantly on cellular defense reactions in which invading microorganisms are encapsulated by blood cells or phagocytosed [38].

The Methanol extract was more active and showed antibacterial activity at lower concentrations. Most familiar active antimicrobial compounds are water insoluble and thus organic solvent extracts have been found more potent. Overall results of screening showed gut tissues (*Perna viridis, Nerita albicilla* and *Ozius rugulosus*) maximally kill pathogens. Sea animals are mainly composed of protein and gut region has more protein content rather than other body parts. As discussed above antibacterial activity in invertebrates is possibly due to action of antibacterial peptides.

A. baumannii has appeared as a highly worrying pathogen for many societies universally. The wide-ranging potential and the clinical severity of *A. baumannii* infections are predominantly related to multidrug-resistant (MDR) strains [39].

Marine invertebrates have developed an effective use of their innate immune system to defend against pathogenic attack by microorganisms. Marine invertebrate antimicrobial peptides are underdeveloped and offer the prospect for a scope of research on antimicrobial peptides.

The biological activity of an extract of marine organisms or insulated compounds could be evaluated in several ways. Due to inadequate quantity of the material normally available at the start and high cost of biological testing, it is difficult in any laboratory to study all combination of drug-animal interaction, to uncover the drug prospective of a material.

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