

Original Research Article

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Scanning Electron Microscopy Analysis of Haemolymph Extract using *Enterococcus faecalis* Drug Resistant Isolates

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ABSTRACT

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Marine organisms are a great source of bioactive substances. Due to the increasing number of structurally unique and biomedically intriguing natural products that are being isolated from marine sources, the area of marine natural products has been rising. In the present study the antimicrobial activity of the mud crab (*Scylla serrata*) was investigated. In this study mud crab (*Scylla serrata*) was collected from mangroves of Karwar, west coast of Karnataka-India, and haemolymph was extracted and examined for bioactivity against *E. faecalis* strains. The antibiotic gentamycin was used along with the least dilution of 10 μ l along with the strains. The haemolymph extract was added of dilution 20 μ l and the pure culture was of 20 μ l respectively. The results showed that the size of the colony as compared to the antibiotic gentamycin and haemolymph extract with *E. faecalis* strains were showing decreased in the cell size as compared to the standard culture (*E. faecalis*) they appeared in the scanning electron microscopic images. Hence the present study shows that the haemolymph of the mud crab has potential antibiotics.

Introduction

Marine invertebrates on innate immune mechanism that include both humoral and cellular responses. Clinical uses of antimicrobial compound were suggested many years ago by several author. However, not much pharmaceutical products as

active compounds have as yet been approved for clinical use as prophylactic or therapeutic drugs against bacterial infections. Some species of the mud crabs have shown antimicrobial activities useful in the biomedical area. Marine organisms are rich sources of structurally diverse bioactivities. They offer enormous resources of functional

materials (Priya Rethna *et al.*, 2015). As most of work on haemolymph as antibacterial has been confine to marine crabs, therefore, presently an attempt has been made to study the antibacterial activity of haemolymph in mud crab (*Scylla serrata*) on *E. faecalis* strain. Our study will set a venture for future research on freshwater crabs as potential candidate for novel drugs with specific actions (Manhas Priya *et al.*, 2017). The innate immune response of marine life is based on humoral and cellular mechanisms that are confined to the haemolymph and are used to combat foreign pathogen invasion.

Additionally, melanization, the generation of reactive oxygen and nitrogen species, and the expression of antimicrobial peptides (AMPs) are all components of the humoral immune response. Because they are the first line of defence against a wide range of harmful microorganisms, such as bacteria, fungi, and viruses, AMPs are essential to the function of insects' innate immune systems (Nesa jannatus *et al.*, 2022). It is now commonly accepted that exploring a larger chemical space will be necessary to identify innovative therapies for Gram-positive infections, since the creation of new molecular scaffolds will give antimicrobials additional targets and activity. Additionally, a number of analyses have shown that current antibiotics that are effective against Gram-positive bacteria have quite different chemical characteristics from standard treatments (Smitten Kirsty *et al.*, 2022). AMPs are a possible substitute for treating infections since pathogenic bacteria are becoming more resistant to traditional antibiotics and medications.

The precise mechanism of action of AMPs remains unclear despite a decade of intensive research. The findings show that to get precise mechanistic insights into peptide function, TEM and SEM are required, together with suitable sample preparation procedures (Hartmannmereike *et al.*, 2010).

The majority of published studies on haemolymph peptides and ion homeostasis have used larval

models. This is because up to 30 mL of haemolymph can be easily collected from a single mud crab of *Scylla serrata*. Due to their mobility, body size and relatively high amount of haemolymph volume, can be collected from mud crabs. Those studies that have measured haemolymph properties directly in mud crabs have done so by pooling minute haemolymph samples from multiple individuals (Macmillan A. Heath and Hughson N. Bryon, 2014). The dirt-scrab Because of its great economic significance, *Scylla* spp. is a crustacean fisheries commodity with bright futures.

The great market demand for crabs is mostly due to the strong nutritional content and delicious taste of these crabs. Popularly sold crab items include soft-shell crabs, frozen crab meat, fresh (living) crabs, and crab eggs. (Hasnidar and Tamsil, 2019). Mud crab's enormous size and great nutritional content have made them more and more popular. They are a vital source of protein for both marine life and humans (Kankamol Chantana and Salaenoi Jintana, 2018). Numerous investigations have been carried out on the biological activities of mud crabs, particularly on antioxidant qualities and antimicrobial peptides (AMPs), in addition to the nutritional perspective. For marine invertebrate immune systems to shield cells from oxidative stress or bacterial invasion, certain defence chemicals must be present. Researchers have been studying marine derived peptides because of their many functional qualities that can be used in the food sector and in nutraceuticals (Yusof Wan Roslina Wan and Ahmad mokhtarnoorasmin, 2020). Antibacterial peptides can also be induced in epidermal cells in response to wounding or infection in the cuticles (Fredrick Sylvester and Ravichandran, 2012). The capacity to investigate surface features at a comparatively high resolution is a unique feature of scanning electron microscopy, which is especially helpful for examining the effects of antibiotics and haemolymph filtrate that coexist with drug-resistant isolates. The current investigation outlines how haemolymph filtrate can be used to treat *E. faecalis* infections.

Materials and Methods

Bacterial strains

Clinical samples (urine samples) were collected from Gulbarga region and isolated on bile asculinazide agar medium incubated it for overnight at 37°C. The growth was observed on the agar plates and were characterized by gram's staining and biochemical test. The confirmed strains were preserved as glycerol stocks and kept it at -18°C for until further use.

Extraction of haemolymph

After the mud crabs (*Scylla serrata*) were collected haemolymph was collected by breaking up each walking and swimming legs of the crab with a fine sterile scissor. The hemolymph is then drawn out with a syringe into 15 to 50 ml of the brown conical tube and stored at -18°C for further use.

Filtration of haemolymph

The crude sample of haemolymph extract was collected in the 15ml of conical tube. The extract was then filtrated by using 0.22-µm syringe filter. Separated the crude sample and the filtrate sample of the hemolymph and placed in two different conical tubes and stored it at -18°C until further use.

Scanning Electron Microscope

The selected isolates of *E.faecalis* were grown in BHI media with haemolymph and standard gentamycin antibiotic. The bacterial cells from each culture were recovered by centrifugation at 6000 rev/min and the cells were washed twice with potassium phosphate buffer (50 mM, pH 7.0). Bacterial cells were then fixed by immersing in 2.5% glutaraldehyde in potassium phosphate buffer (50 mM, pH 7) for overnight at 4°C. Then the specimens were washed twice with buffer and dehydrated by ethanol series (v/v) ranging from 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% and stored in 100% ethanol. For SEM, the specimens

were dried to a critical point, coated with gold, and examined with an S-200C scanning electron microscope compared with a control strain.

Results and Discussion

Bacterial strains

Total 72 clinical samples have been collected from the various hospitals and diagnostic centres of Gulbarga region out of which 40 samples have showed positive growth on the agar plates. Later which were confirmed by gram's staining and biochemical test respectively.

Extraction of haemolymph

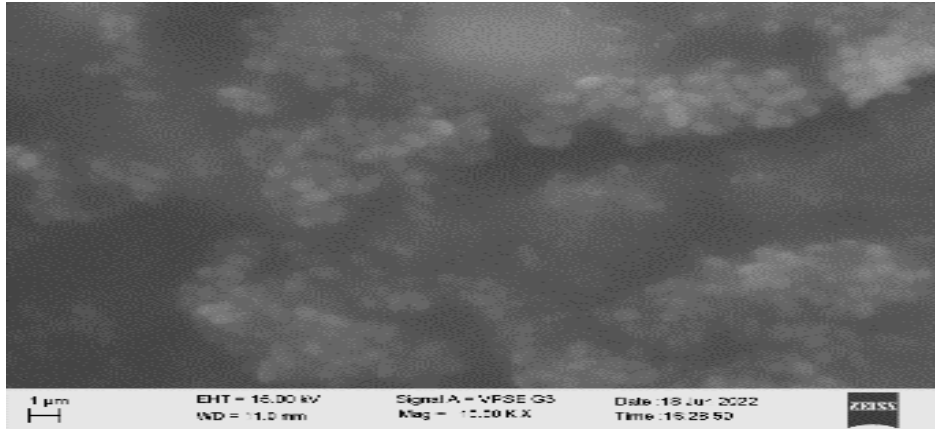
50ml of crude extract of haemolymph was collected from the 20 mud crabs 10 from the male and other 10 from the female the healthy and disease-free crabs have been collected for the extraction of haemolymph. The collected extract was transferred into 50ml of conical tubes and stored it until further use at -18°C.

Scanning Electron Microscope

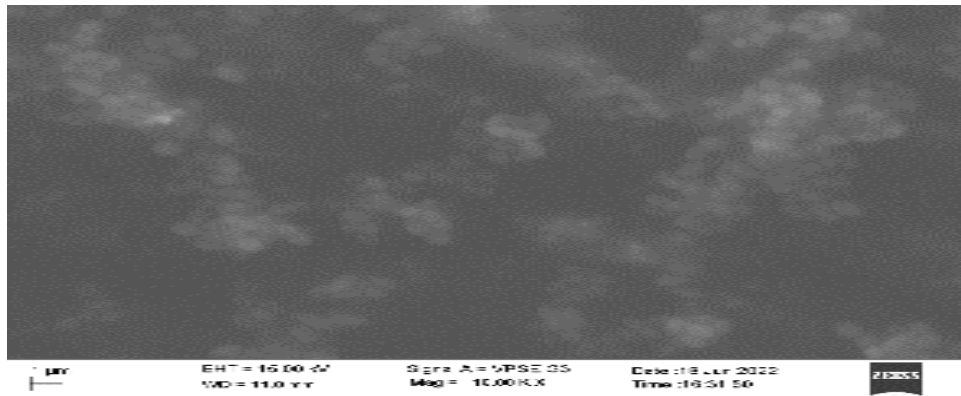
The results of the scanning electron microscopy (SEM) analysis of the cell morphology of the *E. faecalis* isolates showed that in the presence of the antibiotic (gentamycin), the strains' cell morphologies showed 1.3 cm in length and 0.9 cm in width. When the AJEF strains were exposed to antimicrobial peptides, they displayed dimensions of 1.2 cm and 0.9 cm. The identical strains measured 1.4 cm in length and 1.4 cm in width when used as a control with simply culture (fig 01). Due to their potential for pharmacological use, natural compounds' bioactivity has received a lot of attention in recent years. The moon, earth, and sun form an angle of 90° during the half-moon phases I and II. Because the moon and sun are pulling at right angles to each other, the combined gravitational force they exert on the earth is at its weakest, resulting in lower tidal range and weaker tidal forces (neap tides).

Fig.1 SEM images of *E. faecalis* samples when treated with AMP against Standard antibiotic Gentamycin [A] *E. faecalis* Control, [B] Treated with AMP, [C] Treated with std Gentamycin. As compared to the *E. faecalis* control the size of the bacteria decreases when it's treated with Amp samples and std antibiotic gentamycin.

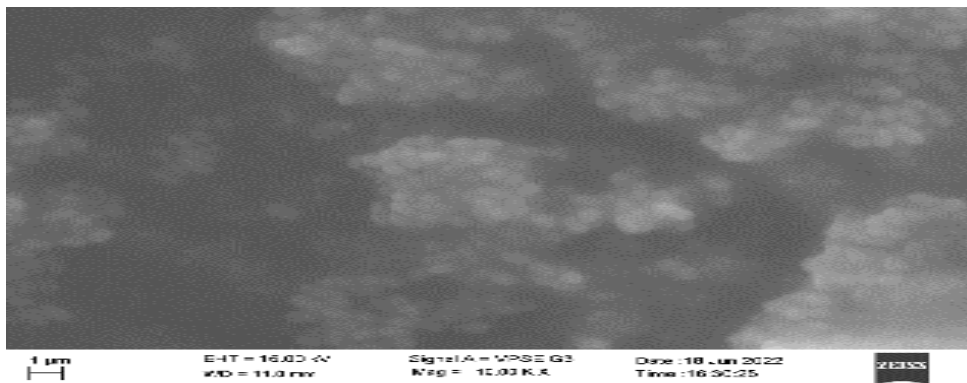
A



B



C



The findings show that these circumstances caused the crabs to produce their highest levels of ecdysteroid hormones and caused moulting (ecdysis). (Hasnidar and Tamsil, 2019). The haemolymph of the mud crab *Scylla serrata* shown antibacterial efficacy in the current investigation against Gram-positive bacterial strains. A similar result was observed with the haemolymph of some brachyuran crabs against clinical pathogens. The main effector molecules of marine immunity are AMPs.

The coastal area of Digha offers a highly promising home for a variety of estuarine and marine crab species. From the research region, a total of 65 species of marine crabs from 36 genera and 13 families were identified. (Srivastava, 2017). Because the mud crabs belonging to the genus *Scylla* are not well identified taxonomically, the taxonomy of these species, which are present in Bangladesh's coastal regions, has become critically important. (Rouf Abdur Mohummad *et al.*, 2016). The Bangladeshi districts of Bagerhat and Satkhira were the study's locations. These districts accounted for 31.45% of Bangladesh's total mud crab production. (Sajan Khan Hayder Md *et al.*, 2021). In this work, 140 mud crabs (*Scylla olivacea*, Herbs, 1976) in total were used. The experimental mud crabs varied in size, with carapace widths between 720 and 800 mm and weights between 90 and 100 g. (Hasnidar and Tamsil, 2019).

The current study is based on the search for antimicrobial peptides haemolymph extraction in mud crab (*Scylla serrata*) that was gathered from the Karwar coastal region India. AMPs screened from crabs possess broad spectrum of biomedically intriguing natural products that are being isolated from marine sources with the potential to avoid antibiotics resistance mechanism. Our study revealed that haemolymph extract from the mud crab has the potential for resistant bacteria as it was compared with std gentamycin along with AMPs. The mud crab showed decrease in both gut-weight and silk-gland-weight due to modulation in feeding behaviour. The filtered extract haemolymph fraction

screened from the mud crab found to be active against *E. faecalis*. As a results from SEM analysis, the cell morphology of *E faecalis* when treated with the std antibiotic gentamycin, *E faecalis* when treated with haemolymph extract (filtrate) and the control *E. faecalis* as standard. Our study reveals that we find that bacteria have developed an adaptive response to antibiotic stress and that the haemolymph filtrate protects against drug-resistant isolates of *E. faecalis*. Given that antimicrobial peptides can treat diseases or infections, this could be a concerning situation.

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