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# Antibacterial Activity from the Haemolymph of Some Brachyuran Crabs

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**Abstract:** Antibiotic research is particularly critical today because of the rise in cases of classic infections diseases which have enjoyed resurgence due to the phenomenon of multi-drug resistance. Five species of crabs were collected from different areas along the Muthupet mangrove region. The results of the present study shows maximum antibacterial activity in the haemolymph of *Dromidiopsis abrolhensis* against *Escherichia coli* and minimum zone of inhibition was observed in the haemolyph of *Scylla serrata* against *Klebsiella oxytoca* in the purified sample. Among the crude and purified haemolymph samples maximum activity was noticed in the crude extracts than purified. On the basis of TLC observations pink spots indicating the presence of amino acids and peptides. Matrix – assisted laser – deionization – of flight (MALDI MS) resulted a number of peaks were detected in the mass/charge range spanning from 579 to 2461 in *S. serrata,* 656 to 845 in *Sesarma brockii,* 1536 to 3256 in *Portunus pelagicus*, 579 to 3659 in crude *D.abrolhensis* and 579 to 1621 in purified *D.abrolhensis*. Among the five selected species, peaks corresponding to low molecular mass compounds were obtained in the three species viz., *Scylla tranquebarica, S.serrata, P.pelagicus* and *D.abrolhensis*. The present investigation was taken up to study the antibacterial activity of crude and purified haemolymph extracts of five dominant mangrove crabs. The mangrove crabs would be a good source of antimicrobial agents and would replace the existing inadequate and cost effective antibiotics.

**Key words:** Crab, Haemolymph, Mangrove, Antibacterial, TLC, MALDI

## INTRODUCTION

Marine organisms represent an excellent source for bioactive compounds. The natural compounds currently under clinical trails are very limited and there is vast potential for the discovery of more potent drugs from the seas. The search for antimicrobial agents has taken direction in developed countries. Crabs which are widely distributed throughout the world have many representatives in the marine and mangrove ecosystems [1-6]. Some mangrove crabs have shown pronounced actives, useful in the biomedical area. The spread of antibiotic resistances and the appearance of multiple-antibiotic-resistant pathogenic bacteria complicate medical treatment of bacterial infections. Researchers have engaged themselves in continuing quest for new and effective antibiotics from natural sources such as plants, invertebrates, etc. to fill the place of the old ones. The present investigation was taken up to study the antibacterial activity of crude and purified haemolymph extracts from the five different mangrove crabs and active samples are taken for the TLC and MALDI studies.

## MATERIALS AND METHODS

**Collection and Preservation of Haemolymph:** Five species of crabs were collected from different areas along the Muthupet mangrove region (Southeast coast of India). Healthy male and female animals at different stages of development were used throughout for experimental purposes and each animal was subjected to a single bleed collections were being done at the time of use.

Haemolymph were collected by cutting each walking legs of the animal with a fine sterile scissor. To avoid hemocyte degranulation and coagulation, the haemolymph was collected in the presence of sodium citrate buffer, pH 4.6 (2:1, V/V). Equal volume of physiological saline (0.85%, NaCl, w/v) was added to it. To remove hemocytes from the haemolymph was centrifuged at 2000rpm for 15min at 4°C. Supernatant were collected by aspirating and stored at 4°C until use.

**Bacterial Strains Used:** Antibacterial activity of crabs haemolymph was determined against 10 bacterial strains

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viz.,. *Pseudomonas aeruginosa, Streptococcus pyogenes, Staphylococcus aureus, Bacillus* sp, *Escherichia coli, Salmonella typhi, Proteus mirabilis, Vibrio cholerae, Klebsiella pneumoniae* and *K. oxytoca.* These pathogens strains were obtained from the department of medical microbiology (Raja Muthiyah Medical College) Annamalai University, Annamalai Nagar.

**Anti Bacterial Assay:** The spectrum of antibacterial activity was studied using as test agent a range of 10 different strain of human pathogenic gram positive and gram-negative bacteria of which there were two antibiotic agents (Ampicillin and Cephalexin).

Invitro antibacterial assay was carried out by disc diffusion technique [7] whatman No. 1 filter paper discs with 4mm diameter were impregnated with known amount test samples of the crabs haemolymph and positive control contained (250mg) of a stand antibiotic disc. Negative controls not comprised sterile disc only. The impregnated discs along with control (incorporated with solvent alone) were kept at the center of Agar Plates, seeded with test bacterial cultures. After incubation at room temperature (37°C) for 24 hrs. Antibacterial activity was expressed in terms of diameter of Zone of inhibition was measured in mm using caliper or a scale and recorded.

**Thin-Layer Chromatography (TLC):** Since in the present investigation, peptides were compounds of interest, ninhydrin was used as detecting agent. This reagent is specific for amines. TLC plate is first developed in a suitable solvent system, dried at room temperature, sparyed with ninhydrin and heated at 100°C in oven for few minutes till the coloured spots are visible. Checked for pink spots (amino acids and amines).

As the soluble fractions showed ninhydrin positive spots they were subjected to purification by gel permeation chromatography on sephadex LH 20 using methanol as eluent and monitored by TLC. Diagnostic thin layer chromatography was performed on methanol and chloroform extracts were also spotted and plates developed in varying proportions. Detection was done with the color reagent ninhydrin, specific for detecting the compounds.

**Column Chromatography**

**Gel Filtration Chromatography:** Size exclusion chromatography or gel filtration is an important technique in which the support or gel is a neutral porous material that allows molecules of different sizes to penetrate into the gel to different extents. In general, molecules will be eluted in the reverse order of their molecular size, although for smaller molecules, other factors, such as polarity, will play a role. Sephadex LH-20, obtained by alkylations of most of the hydroxyl groups of sephadex G-25, seems to have become the most popular of the hydrophilic gel in the isolation of natural products.

**MALDI (Matrix-assisted Laser-Deionization-Time-of-Flight) MS:** MALDI-TOF MS was performed on an Ultraflex MALDI TOF/TOF (Bruker Daltonics) instrument. The Mass spectrosopic measurements were performed on two different ionization matrix's viz., 2,5-dihydroxybenzoic acid (DHB) and α-cyno-4-hydroxycinnamic acid (CCA).

## RESULTS

**Antibacterial Activity:** In the present study ten bacterial strains was used against the crude and pure haemolymph of five brachyuran crabs for antibacterial assay. Among the tested samples maximum activity (6mm) was recorded in the crude haemolymph of the crab *D.abrolhensis* against *E.coli* and minimumn activity (1.5mm) was recorded in the purified hemolymph of *S.serrata* against *K.oxytoca* is shown in (Table 1-4).

The crude and purified sample of *D. abrolhensis* shows maximum zone of inhibition against *E.coli* and minimum zone of inhibition of crude extracts was reported in *Streptococcus* and *S.aureus* strains (2mm) and purified samples minimum zone of inhibition was recorded in *S.aureus* and *K.pneumoniae* strains (2mm). But in the crude and purified haemolymph sample of *Sesarma brockii* there is no activity against the ten tested pathogenic strains.

**Thin-Layer Chromatography (TLC):** TLC profiling was done for all the five fractions (*S.tranquebarica, S.serrata, P. pelagicus, S.brockii* and *D.abrolhensis,*) in two different solvent systems A and B. The solvent system A consisted of methanol: chloroform (1:9) and B was a combination of butanol, acetic acid and water (B: A: W) in proportions of 5:1:4. The plates when developed in both the solvent systems showed light pink spots when sprayed in ninhydrin. The plate with fractions developed in BAW as the solvent system and sprayed with ninhydrin, showing pink spots indicating the presence of amino acids and peptides.

Table 1: Antibacterial activity of *Scylla tranquebarica*

| S.No | Name of the Bacteria | Gram +Ve/-Ve | Activity | Zone of inhibition (mm in diameter) procured by induced haemolymph: Crude | Purified |
|---|---|---|---|---|---|
| 1. | *Pseudomonas aeruginosa* | -- | +Ve | 3..5 | 3 |
| 2. | *Streptococcus pyogenes* | -- | -Ve | - | - |
| 3. | *Staphylococcus aureus* | + | +Ve | 2..5 | 2 |
| 4. | *Bacillus* sp | + | +Ve | 4..5 | 4 |
| 5. | *Escherichia coli* | -- | +Ve | 5 | 5..5 |
| 6 | *Salmonella typhi* | -Ve | +Ve | 5 | 5 |
| 7 | *Proteus mirabilis* | -Ve | -Ve | 4 | 4..5 |
| 8 | *Vibrio cholerae* | -Ve | +Ve | 5 | 4 |
| 9 | *Klebsiella pneumoniae* | -Ve | +Ve | 3 | 2..5 |
| 10 | *Klebsiella oxytoca* | -Ve | -Ve | - | - |

Table 2: Antibacterial activity of *Scylla serrata*

| S.No | Name of the Bacteria | Gram +Ve/-Ve | Activity | Zone of inhibition (mm in diameter) procured by induced haemolymph: Crude | Purified |
|---|---|---|---|---|---|
| 1 | *Pseudomonas aeruginosa* | +Ve | +Ve | 4..5 | 4 |
| 2 | *Streptococcus pyogenes* | +Ve | +Ve | 5 | 4 |
| 3 | *Staphylococcus aureus* | -Ve | +Ve | 4 | 3..5 |
| 4 | *Bacillus* sp | -Ve | +Ve | 3 | 3 |
| 5 | *Escherichia coli* | -Ve | +Ve | 4 | 3..5 |
| 6 | *Salmonella typhi* | -Ve | +Ve | 5. | 5..5 |
| 7 | *Proteus mirabilis* | -Ve | +Ve | 4..5 | 3 |
| 8 | *Vibrio cholerae* | -Ve | +Ve | 5..5 | 5 |
| 9 | *Klebsiella pneumoniae* | -Ve | +Ve | 5 | 4 |
| 10 | *Klebsiella oxytoca* | -Ve | +Ve | 2 | 1..5 |

Table 3: Antibacterial activity of *Portunus pelagicus*

| S.No | Name of the Bacteria | Gram +Ve/-Ve | Activity | Zone of inhibition mm in diameter) procured by induced haemolymph: Crude | Purified |
|---|---|---|---|---|---|
| 1 | *Pseudomonas aeruginosa* | +Ve | -Ve | - | - |
| 2 | *Streptococcus pyogenes* | +Ve | -Ve | - | - |
| 3 | *Staphylococcus aureus* | -Ve | -Ve | - | - |
| 4 | *Bacillus* sp | -Ve | -Ve | - | - |
| 5 | *Escherichia coli* | -Ve | -Ve | 4 | 3..5 |
| 6 | *Salmonella typhi* | -Ve | -Ve | - | - |
| 7 | *Proteus mirabilis* | -Ve | -Ve | - | - |
| 8 | *Vibrio cholerae* | -Ve | -Ve | 2..5 | 2 |
| 9 | *Klebsiella pneumoniae* | -Ve | -Ve | 3 | 3 |
| 10 | *Klebsiella oxytoca* | -Ve | -Ve | - | - |

Table 4: Antibacterial activity of *Dromidiopsis abrolhensis*

| S.No | Name of the Bacteria | Gram +Ve/-Ve | Activity | Zone of inhibition (mm in diameter) procured by induced haemolymph: Crude | Purified |
|---|---|---|---|---|---|
| 1 | *Pseudomonas aeruginosa* | +Ve | -Ve | 4..5 | 3..5 |
| 2 | *Streptococcus pyogenes* | +Ve | -Ve | 2 | - |
| 3 | *Staphylococcus aureus* | -Ve | -Ve | 2 | 3 |
| 4 | *Bacillus* sp | -Ve | -Ve | 4..5 | 4 |
| 5 | *Escherichia coli* | -Ve | +Ve | 6 | 5..5 |
| 6 | *Salmonella typhi* | -Ve | -Ve | 5 | 5 |
| 7 | *Proteus mirabilis* | -Ve | -Ve | 4..5 | 4 |
| 8 | *Vibrio cholerae* | -Ve | -Ve | 5 | 4 |
| 9 | *Klebsiella pneumoniae* | -Ve | +Ve | 3..5 | 3 |
| 10 | *Klebsiella oxytoca* | -Ve | -Ve | - | - |

Note: Where numbers indicate zone of inhibition, minus sign (-) indicates no antibacterial activity, numbers indicated marked inhibition zone, +ve indicates positive and -ve means negative.

Table 5: Multiple spectra for different extraction of samples

| S.No | Crab species | Matrix | No. of peaks | Common peaks |
|---|---|---|---|---|
| 1. | *S.tranquebarica,* | CC A | 15 | 579.282<br>871.431 |
| 2. | *S.serrata* | CC A | 16 | 579.300<br>707.416 |
| 3. | *P.pelagicus* | CC A | 13 | 1536 |
| 4. | *S.brockii* | DH B | 4 | 656.064<br>782.591<br>845.185 |
| 5 | *D.abrolhensis* (crude) | DH B | 19 | 579.337<br>707.447<br>872.490 |
| 6 | *D.abrolhensis* (purified) | DH B | 10 | 579.344<br>707.456<br>872.497 |

CCA: Dihydroxybenzoic acid DHB: Cyano hydroxycinnamic acid

**MALDI:** As shown in (Table 5), matrix – assisted laser – deionization – of flight (MALDI MS) profile of a small drop of the raw sample and purified sample of the haemolymph of crabs in Dihydroxybenzoic acid (CCA) and Cyano hydroxycinnamic acid (DHB) ionization matrixes revealed a peak at 579 m/z and 707m/z which was common in almost all the samples. Likewise m/z 872 in crab species *D.abrolhensis* was also showing in both raw sample and purified sample. In *S.brockii* species prominent peak was observed at the m/z 656. Among the five selected species, peaks corresponding to low

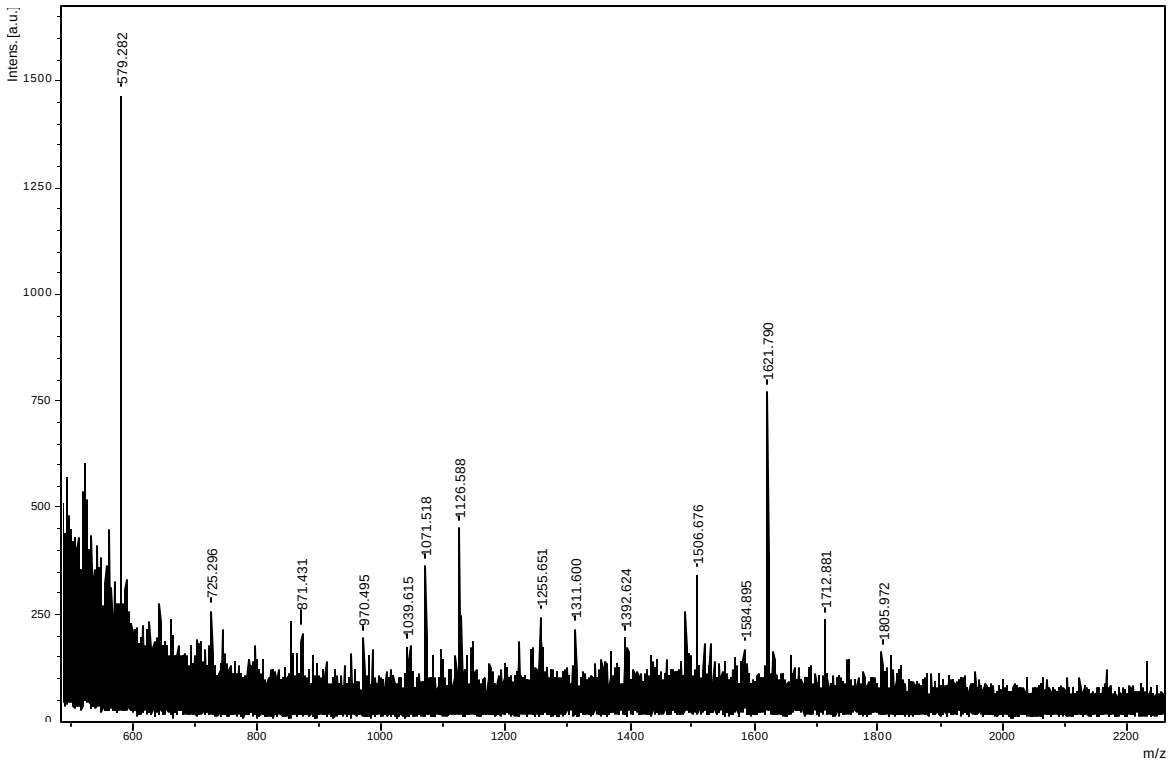


Fig. 1: Multiple spectra report of *S.tranquebarica* in direct method

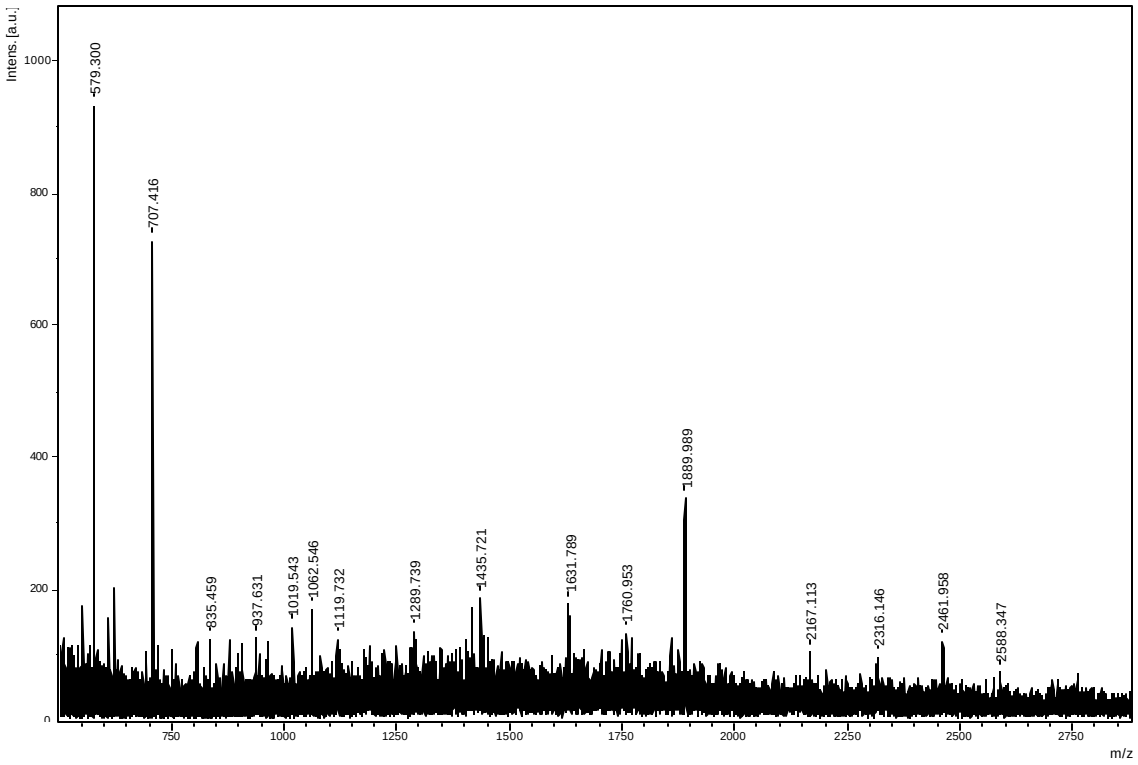


Fig. 2: Multiple spectra report of *S.serrata* in direct method (CCA matrix)

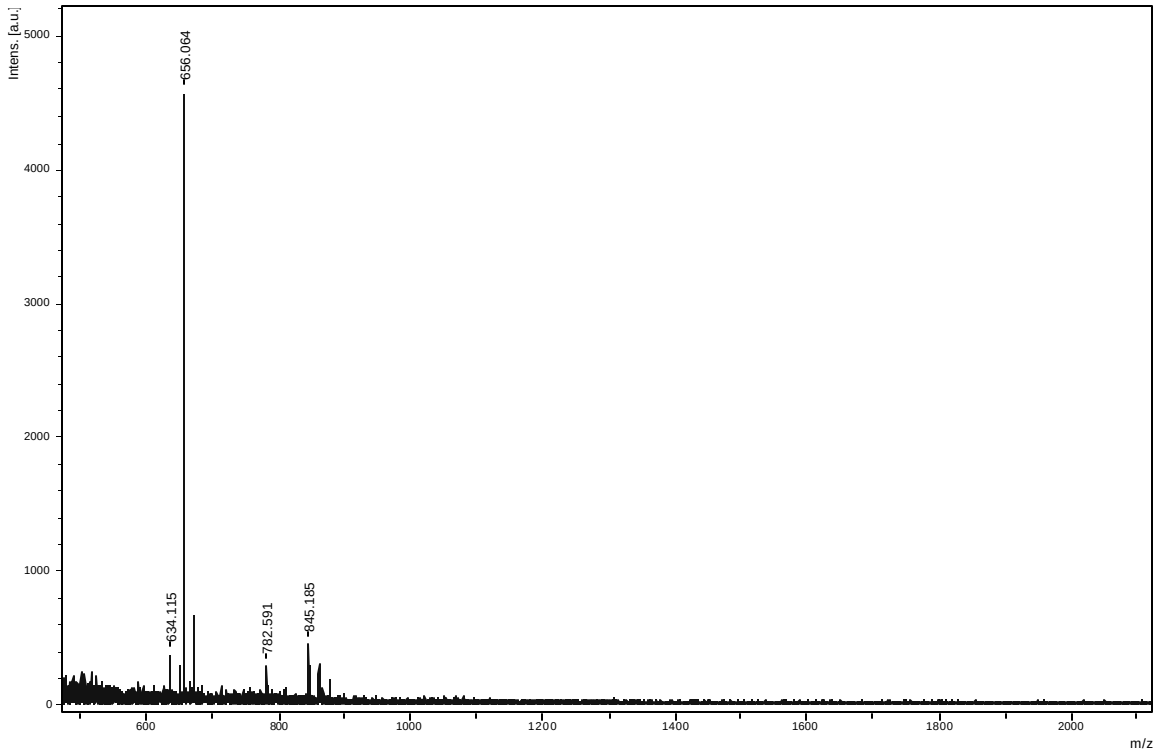


Fig. 3: Multiple spectra report of *S.brockii* in direct method (DHB-matrix)

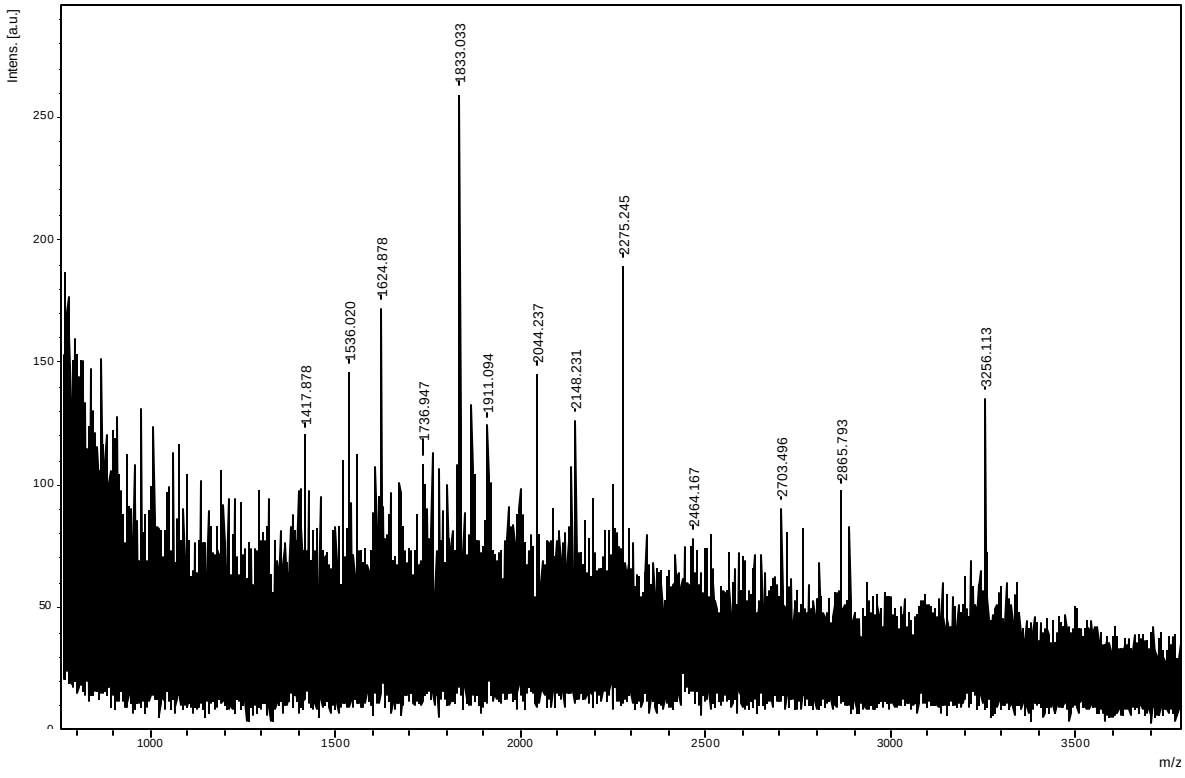


Fig. 4: Multiple spectra report of *P.pelagicus* in CCA-matrix

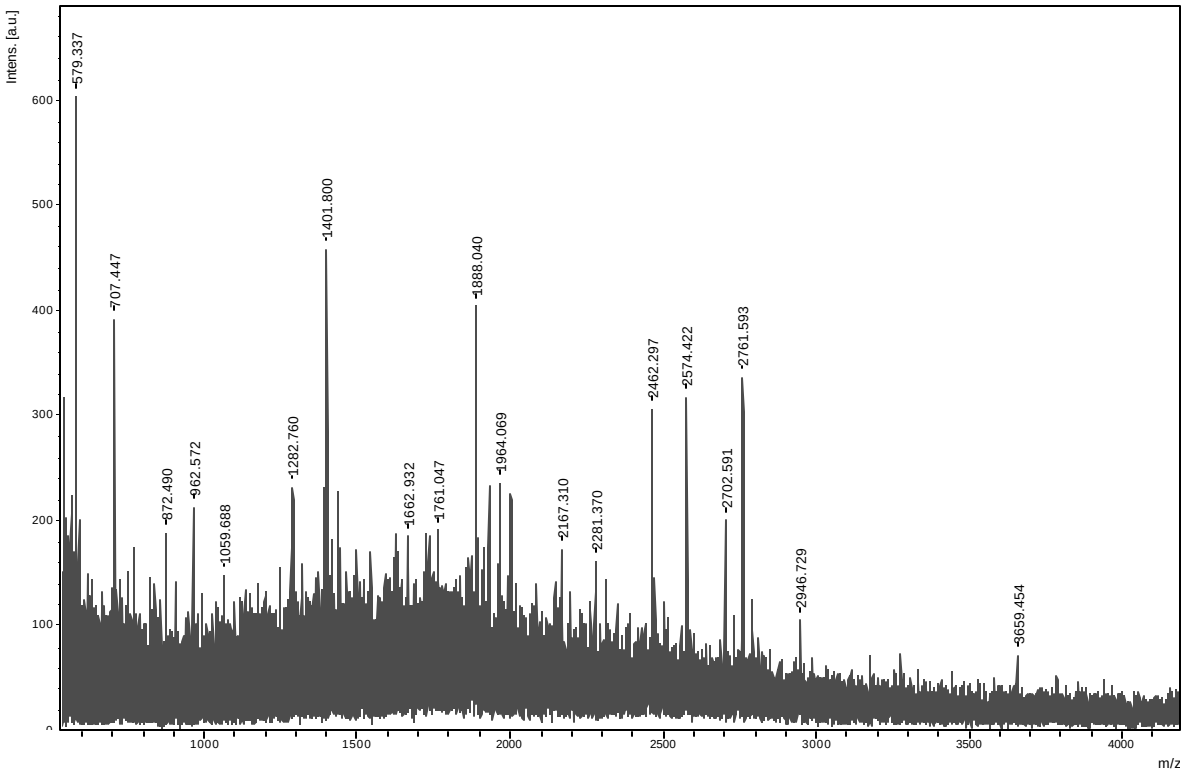


Fig. 5: Multiple spectra report of crude sample of *D.abrolhensis* in direct method (DHB- matrix)

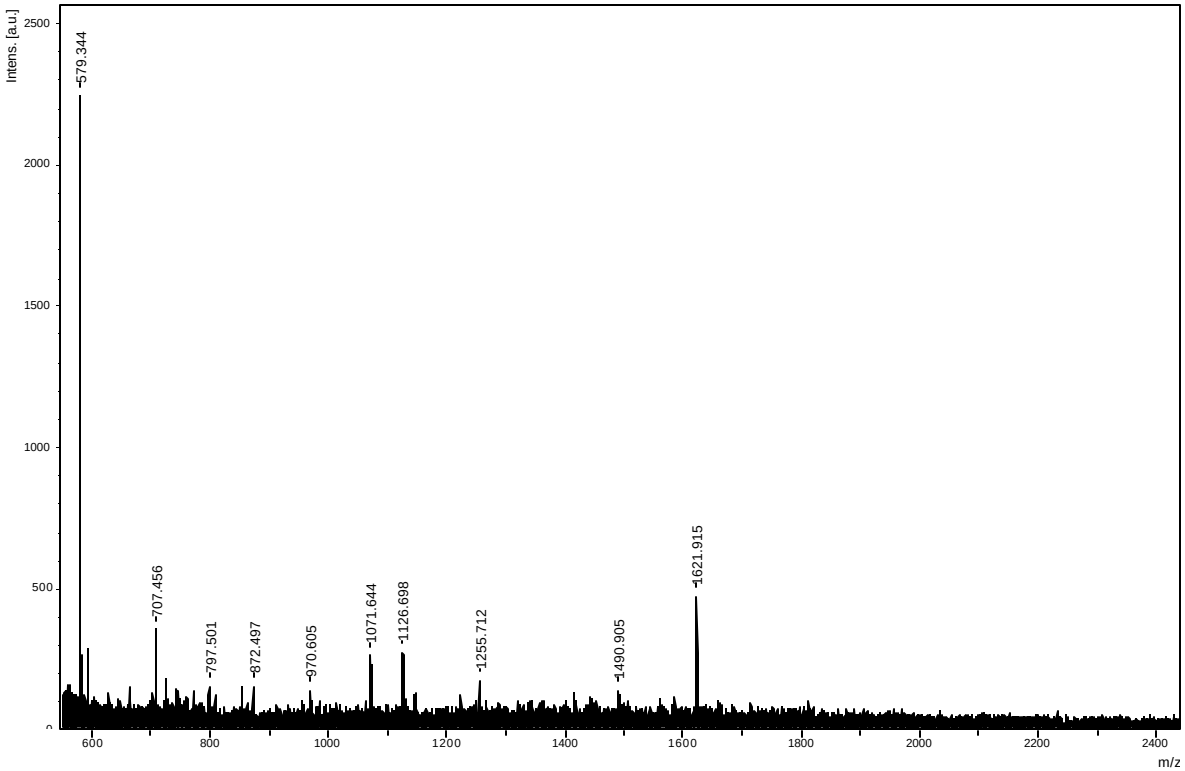


Fig. 6: Multiple spectra report of purified *D. abrolhensis* in DHB-matrix

molecular mass compounds were obtained in the three species viz., *S.tranquebarica, S.serrata, P.pelagicus* and *D.abrolhensis*. A number of peaks were detected in the mass/charge range spanning from 579 to 2461 in *S.serrata,* 656 to 845 in *S.brockii,* 1536 to 3256 in *P.pelagicus*, 579 to 3659 in crude *D.abrolhensis* and 579 to 1621 in purified *D.abrolhensis*. The results were displayed in (Fig. 1-6) and are summarized in (Table 5).

## DISCUSSION

The presence of naturally occurring in the haemolymph of several crustaceans has been well known since the beginning of the 20th century [8]. The decapod crustaceans, it is known that environmental changes may affect the immune ability to susceptibility against pathogen infection. In the present investigation, haemolymph were collected from five different mangrove crabs viz. *S. tranquebarica*, *S. serrata*, *P. pelagicus, S. brockii* and *D. abrolhensis* were subjected to antibacterial assay.

The presence of antimicrobial compounds has been reported in crustacean species including the crabs *Carcinus maenas* [9] and *Callinectes sapidus* [10] but, to date no data were available. In the present study, the haemolymph showed antimicrobial activity against a range of different strains of gram positive and gram negative bacteria including few antibiotic resistant strains. The results suggest that mangrove crab can produce antimicrobial substances instantly to combat bacterial infection. Induction of antibacterial compounds was also observed in case of sarcotoxin I [11]and sapecin [12] in Sarcophaga peregrine, moricin [13], lebocin [14] and ceropin-B [15] in *B. mori*. As the haemolymph showed antibacterial compounds were secreted in response to immunization. Similar observations were also found [16] in *Tachypleus tridentatus* [17], *B.mori* [18] and *Hyalophora ceropia*.

As described for Limulus [19] and to some extent for mammalian antimicrobial peptides, some of the penaeidens stored in blood cells appear to be released into haemolymph upon stimulation. Actually microbial stimulation is known to trigger hemocyte of degranulation as one of the most immediate hemocytic reactions in crustaceans [20, 21]and in the freshwater crayfish *Pacifastacus leniusculus*, degranulation was shown to be associated with a rapid decrease in RNA and protein synthesis in granular cells.

Among the crude and purified haemolymph samples maximum activity was noticed in the crude extracts than purified. Maximum inhibition of crude sample is the common phenomena because the preservative and buffer mixture in the crab haemolymph will also some time inhibit the growth of bacterial strains. The haemolymph also inhibited growth of antibiotic resistant strains tested. It is an interesting finding that crabs, being marine animal has the ability to dispose the bacteria upon infection. As the bacterium is a human pathogen, it is important that sea water should be free from this type of bacteria. The results of MALDI- MS revealed a peak at 579 m/z and 707m/z which was common in almost all the samples. Among the five selected species, peaks corresponding to low molecular mass compounds were obtained in the four species viz., *S.tranquebarica, S.serrata, P.pelagicus* and *D.abrolhensis*. The present study indicates that the haemolymph of mangrove crabs would be a good source of antimicrobial agents and would replace the existing inadequate and cost effective antibiotics.

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