Advances in Biological Research 3 (3-4): 104-109, 2009
ISSN 1992-0067
© IDOSI Publications, 2009

Influence of Crab Haemolymph on Clinical Pathogens

R.M. Anbuchezhian, S. Ravichandran G. Rameshkumar and T.T. Ajithkumar

Centre of Advanced Study in Marine Biology, Annamalai University,
Parangipettai 608 502, India

Abstract: Marine organisms are capable of surviving and growing in habitats of extremes. In crustaceans, the defense system against microbes rests largely on cellular activities performed by hemocytes such as adhension, phagocytosis, encapsulation, nodule formation and melanisation. In the present study, seven crabs species *Charybdis feriata, C. lucifera, C. amboinensis, C. natator, Portunus sanguinolentus, P. pelagicus* and *Dromia dehanni,* were collected from the mouth, oyster and mangrove region of Vellar estuary for the antimicrobial studies. The antibacterial activity was detected maximum zone of inhibition in the haemolymph of *P.pelagicus* against *Staphylococcus aureus* (15mm) where as minimum activity (4mm) was detected in the haemolymph of *P.sanguinolentus*, *C.lucifera C.amboinensis* and *D.dehanni* againsts *K.oxytoca L.vulgaris S.aureus* and *K.pneumonia*. But in antifungal activity the highest zone of inhibition was observed in the haemolymph of *D.dehanni* against *Trichophyton mentagrophytes* (11mm) and the minimum (4mm) was recorded in *C.natator* and *P.sanguinolentus* against *T.mentagrophytes* and *Epidermophyton flococusum.* This study shows that the haemolymph of brachyuran crabs may potential antibiotics.

Key words: Vellar estuary, Crab, Haemolymph, Antibacterial, Antifungal

INTRODUCTION

Marine invertebrates are constantly exposed to high concentrations of microorganisms. In crustaceans, the defense system against microbes rests largely on cellular activities performed by hemocytes such as adhension, phagocytosis, encapsulation, nodule formation and melanisation. The multimeric coagulation and phenoloxidase systems are also considered to be important defenses in these organisms. Other factors described as part of the immune system include agglutinins, hemolysins, lysozyme and antimicrobial factors [1, 2]. During the past two decades, the molecular structures and functions of various defense components that participated in innate immune systems have been established in arthropoda, such as, insects, the horseshoe crab, freshwater crayfish and the protochordata, ascidian. These defense molecules include phenoloxidases, clotting factors, complement factors, lectins, protease inhibitors, antimicrobial peptides, toll receptors and other humoral factors found mainly in haemolymph plasma and hemocytes. These components, which together compose the innate immune system, defend invertebrate from invading bacterial, fungal and viral pathogens.

Antimicrobial activity has been detected in several decapod crustaceans, including lobster, crabs, shrimps and freshwater crayfish [3,4]. The crabs are to be rich source of bioactive compounds, but the researchers carried out so far regarding there pharmacological properties are scanty. Hence the present investigation was taken up to study the antibacterial activity of haemolymph extracts from seven different crabs against 10 bacterial strains and antifungal activity against 8 fungal strains.

MATERIALS AND METHODS

Collection of Animals: In the present study, seven crabs species *Charybdis feriata, C. lucifera, C. amboinensis, C. natator, Portunus sanguinolentus, P. pelagicus* and *Dromia dehanni* were collected from the mouth, oyster and mangrove region of Vellar estuary (Lat 11° 29' N; 79° 46' E), Parangipettai, along the southeast coast of India. Healthy male and female animals at different stages of development were used throughout for experimental purpose and animal was subjected to a single bleed.

Corresponding Author: S. Ravichandran, Centre of Advanced Study in Marine Biology,
Annamalai University, Parangipettai 608 502, India

Collection of Haemolymph: 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.

Microbial Strains Used: Antibacterial activity of crabs haemolymph was determined against 10 bacterial strains viz., *Staphylococcus aureus, Salmonella typhi, S. paratyphi, Klebseilla oxytoca, Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Lactobacillus vulgaris, Vibrio* sp and *K. Pneumoniae* and antifungal activity was determined against 8 fungal strains viz., *Candida albicans, Aspergillus niger, Trichophyton mentagrophytes, Rhizopus* sp, *Epidermophyton flococusum, A. flavus, A. fumigatus, Cryptococcus neoformans.*

Antimicrobial Assay: The spectrum of antimicrobial activity was studied using as test agent a range of 10 different strains of human pathogenic gram positive and gram-negative bacteria and eight different species of fungal pathogen of which there were one antibiotic agent Erythromycin for pathogenic bacteria and Flucanazole for pathogenic fungi.

In vitro antibacterial assay was carried out by disc diffusion technique [5] What man No. 1 filter paper discs with 4mm diameter were impregnated with known amount test samples of the crabs haemolymph and positive control contained a standard 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 and 0.1 ml of 24 hrs old fungal culture separately. After incubation at room temperature (37°C) for 24 hrs for bacterial plates and after 72hrs at 30°C for the fungal plates, antimicrobial activity was expressed in terms of diameter of Zone of inhibition was measured in mm using caliper or a scale and recorded.

RESULTS

Antibacterial Activity: In the present study ten bacterial strains was used against the hemolymph of seven brachyuran crabs for antibacterial activity. Among the tested samples maximum activity (15mm) was recorded in the haemolymph of *P.pelagicus* against *S.aureus* and minimumn activity (4mm) was recorded in the hemolymph of *D. dehanni, P. sanguinolentus, C. luciifera* and *C. amboinensis* against *K. pneumonia, K. oxytoca, L. vulgaris* and *S. aureus* (Fig. 1).

Antibacterial Activity of *P. pelagicus*: The maximum zone of inhibition (15mm) was recorded in the haemolymph of *P.pelagicus* against *S.aureus* and minimum zone of inhibition (6mm) was observed in *P.mirabilis* strain (Fig.1a and 1h). Among the ten pathogenic strains *S.paratyphi* and *E.coli* shown negative activity and rest of them shown positive activity

Antibacterial Activity of D.Dehanni: Antibacterial activity of *D. dehanni* the zone of inhibition varied from 9mm to 4mm. Maximum diameter was noted against *K.oxytoca* and minimum zone of inhibition were recorded against *L.vulgaris* and *K.pneumonia.* (Fig.1c and 1e).

Antibacterial Activity of P.Sanguinolentus: The maximum of 10mm and minimum of 4mm inhibition zones were recorded in the haemolymph of *P. sanguinolentus* against *S.aureus* and *K.oxytoca.* (Fig.1a and 1c).

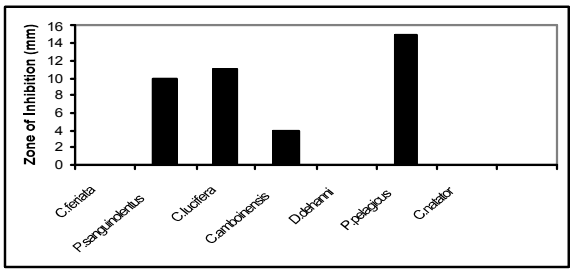
Antibacterial Activity of C.Luciifera: The haemolymph of *C.luciifera* shown maximum inhibition zone (11mm) against *S.aureus* and minimum inhibition zone (4mm) was recorded against *L.vulgaris.* (Fig.1a and 1e).

Antibacterial Activity of C. Ferriata: In the haemolymph of *C. ferriata* shows 5mm of zone of inhibition against *S.typhi* and *K.oxytoca*. (Fig.1b and 1c).

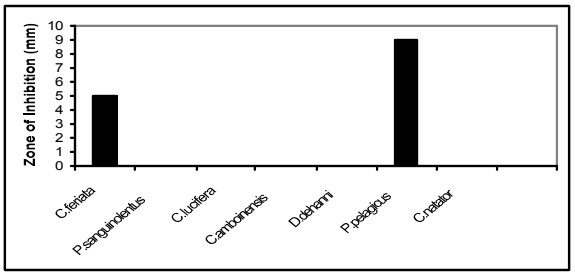
Antibacterial Activity of C. Amboinensis: The haemolymph of *C.amboinensis* was shown maximum inhibition zone (5mm) against *L. vulgaris* and minimum zone of inhibition (4mm) against *S.aureus.* (Fig.1e and 1a).

Antibacterial Activity of C. Natator: Antibacterial activity of *C. natator* haemolymph was not shown any antibacterial activity against any pathogenic bacterial strain.(Fig.1).

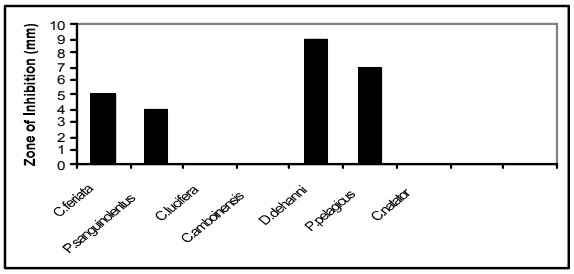
Antibacterial Activity of Positive Control: The antibacterial activity of positive control (Erythromycin) shows activity against almost all the pathogenic bacterial strains. The maximum activity was recorded against *S.aureus* (20mm) and minimum activity was observed against *P. aeruginosa* (15mm).



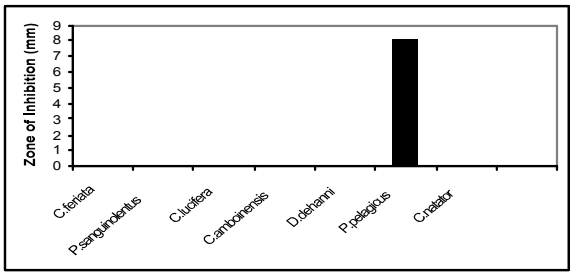
a. Haemolymph of crabs against *S.aureus*



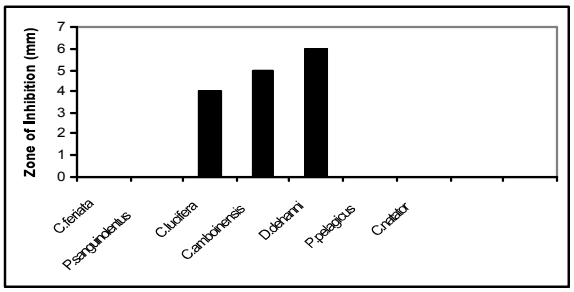
b. Haemolymph of crabs against *S.typhi*



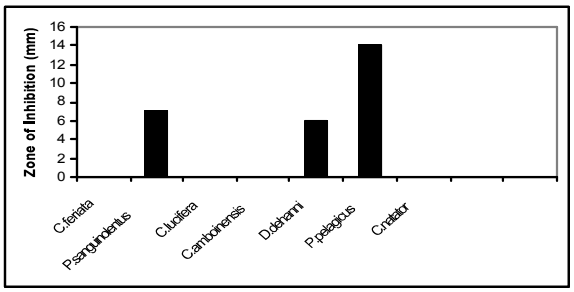
c. Haemolymph of crabs against *K.oxytoca*



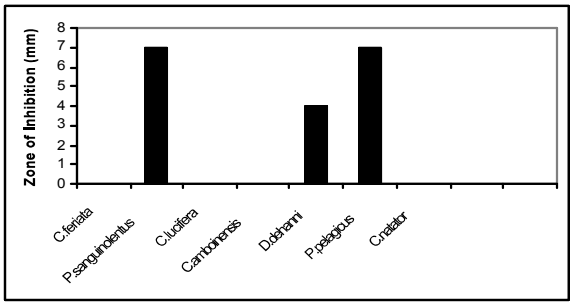
d. Haemolymph of crabs against *P.aeruginosa*



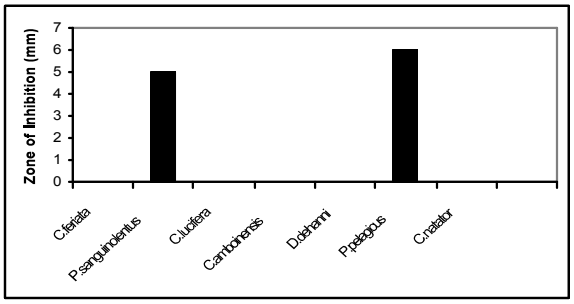
e. Haemolymph of crabs against *L.vulgaris*



f. Haemolymph of crabs against *Vibrio* sp.

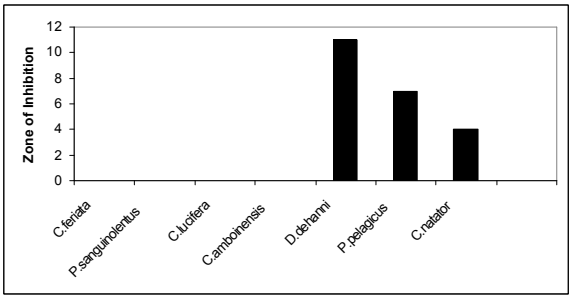


g. Haemolymph of crabs against *K.pneumonia*

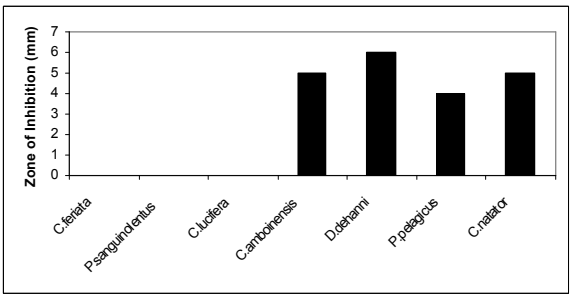


h. Haemolymph of crabs against *P.mirabilis*

Fig. 1: Antibacterial activities of crab haemolymph



a. Haemolymph of crabs against *T.mentagrophytes*



b. Haemolymph of crabs against *E.flococusum*

Fig. 2: Antifungal activities of crab haemolymph

Antifungal Activity: In the present study eight fungal strains was tested against the haemolymph of seven brachyuran crabs. Among the tested samples maximum activity (11mm) was showed in the haemolymph of *D.dehanni* against *T. mentagrophytes* and minimumn activity (4mm) was showed in the haemolymph of *P.pelagicus* and *C.natator* against *T. mentagrophytes* and *E.flococusum* (Fig.2).

Antifungal Activity of D. Dehanni: Antifungal activity of the haemolymph of *D.dehanni* against *T. mentagrophytes* was observed maximum zone of inhibition (11mm) and minimum zone of inhibition was noticed in *E. flococusum* (6mm) (Fig.2a and 2b).

Antifungal Activity of P.Pelagicus: The *P.pelagicus* crab haemolymph showed activity against tested fungal strains was observed maximum zone of inhibition in *T. mentagrophytes* (7mm) and minimum zone was recorded against *E.flococusum* (4mm). (Fig.2a and 2b).

Antifungal Activity of C.Natator: Haemolymph of *C.natator* shows antifungal activity was maximum against *E.flococusum* (5mm) and minimum inihibition zone was recorded against *T. mentagrophytes* (4mm). (Fig.2b and 2a).

Antifungal Activity of C.Amboinensis: Antifungal activity of *C.amboinensis* was recorded only against *E.flococusum.* The zone of inhibition was 5mm. Rest of the haemolymph samples did not shown antifungal activity. (Fig.2a).

Antifungal Activity of Positive Control: Antifungal activity of fluconazole shows maximum zone of inhibition (11mm) in *Rhizopus* and minimum zone of inhibition (8mm) was observed against *A.fumigates*.

DISCUSSION

A screening of antimicrobial activity of different crab species was conducted. In the present study the crab haemolymph shown antimicrobial activity against the range of different bacterial strains of both gram positive and gram negative bacteria and pathogenic fungal strains including few antibacterial resistant strains. Previous works shown that marine decapod crustaceans contain factors with antibacterial activity, particularly in the haemolymph and/or the hemocytes. This property seems to be a common feature throughout the order [6]. Antibacterial activity has been previously described in a wide range of crustacean species [4,7,8]. But antimicrobial activity of the present study reported crabs are not reported earlier.

The result suggests that the crab can produce antimicrobial substances instantly to combat microbial infection. 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. Usually it should not be in the water and the peptides can kill more efficiently than the conventional antibiotics.

In arthropods, antimicrobial compounds were mainly studied in chelicerates (Horseshoe crabs) and insects. Their involvement in the defense reaction is quite different in these two groups. In horse shoe crabs, they are mainly synthesized in hemocytes (Blood cells) where they are stored after processing within their cytoplasmic granules [9]. They are believed to be released into haemolymph through regulated exocytosis upon microbial stimulation. The presence of antimicrobial compounds has been reported in crustacean species including the crabs *Carcinus maenas* [10] and *Callinectes sapidus* [11] but, to date no data were available.

Induction of antibacterial compounds were also observed in case of sarcotoxin I [12] and sapecin [13] in sarccophaga peregrine, moricin [14], lebocin [15] and cecropin-B [16] in *Bombyx mori*). As the haemolymph showed antibacterial against both gram positive and gram negative bacteria, It offers to comment that broad spectrum antibacterial compounds where secreted in response to immunizations. Similar observations were also found by [17] in *Tachypleus tridentatus,* [18] in *Bombyx mori*, [19] in *Hyalophora cecropia.* The antimicrobial activity might also be due to factors of innate immune system.

In the present study findings shows maximum antibacterial activity could be detected in the haemolymph of *P.pelagicus* against *S.aureus* (15mm) where as minimum activity was detected in *P.sanguinolentus*, *C.lucifera. Camboinensis* and *D.dehanni* againsts *K.oxytoca L.vulgaris S.aureus* and *K.pneumonia* (4mm). Similar result was observed in the haemolymph of some grapsid crabs against pathogenic fungi and bacteria [20,21].

But in antifungal activity the highest zone of inhibition was observed in the haemolymph of *D.dehanni* against *T. mentagrophtes* (11mm) and the minimum (4mm) was recorded in *C.natator* and *P.sanguinolentus* against *T.mentagrophytes* and *E.flococusum.* These results indicate that crabs have developed a variety of defense molecules in haemolymph against pathogenic microorganisms and the kind of dominating vary among the different species. From the pharmacological point of view it is advantageous, antimicrobial drugs have no side effects. This study shows that a number of marine crabs contain factors with antimicrobial activity in haemolymph. In conclusion in the present study indicates that the haemolymph of crab would be a good source of antimicrobial agents and would replace the existing inadequate and cost effective antibiotics.

ACKNOWLEDGEMENT

The authors are grateful to the Director of CAS in Marine Biology and authorities of Annamalai University for facilities provided.

REFERENCES

1. Smith, V.J. and J.R.S. Chisholm, 1992. Non cellular immunity in crustaceans. Fish-Shellfish Immunol., 2: 1-31.
2. Soderhall, K. and L. Cerenius, 1992. Crustacean immunity. Annual Review of fish Diseases, 2: 3-23.
3. Stewart, J.E. and B.M. Zwicker, 1972. Natural and induced bactericidal activities in the haemolymph of the lobster; *Homarus americanus*: products of hemocyte-plasma interactions. Canadian J. Microbiol., 18: 1499-1509.
4. Noga, E.J., Arroll, T.A. Fan and Zhigin, 1996. Specificity and some physiochemical characteristics of the antibacterial activity from the blue crabs *Callinectes sapidus*. Fish-Shell fish-Immunology, 6(6): 403-12
5. Bauer, A.W., W.M.M. Kirby, J.C. Sherris and M. Turck, 1996. Antibiotic susceptibility testing by a standardized single disc method. American J. Clinical Pathol., 45: 493-496.
6. Haug, T., A.K. Kjuul, E. Sandsdalen and O.B. Styrvold, 2002. Antibacterial activity in four marine crustacean decapods. Fish-shellfish Immunology, 12; 5: 371-85.
7. Jayasankar, V. and T. Subramoniam, 1999. Antibacterial activity of seminal plasma of the mud crab *Scylla serrata* (forskal). J. Experimental Marine Biol. Ecol., 236: 253-259.
8. Chisholm, J.R.S. and V.J. Smith, 1995. Comparison of antibacterial activity in the hemocytes of different crustacean species. Comparative Biochem. Physiol., 110A: 39-45.
9. Iwanaga, S. and S. Kawabata, 1998. Evolution and phylogeny of defense molecules associated with innate immunity in horseshoe crab. Fronties Bioscience, 3: 973-84.
10. Schnapp, D., G.D. Kemp and V.J. Smith, 1996. Purification and characterization of a proline-rich antibacterial peptide, with sequence similarity to bactenecin-7, from the haemocytes of the shore crab, *Carcinus maenas*. Eurobian J. Biochem., 240:532-539.
11. Khoo, L., D. Robinette and E. Noga, 1999. Callinectin, an antibacterial peptide from blue crab, *Callinectes sapidus*, hemocytes. Marine Biotechnology, 1: 44-51.
12. Okada, M. and S. Natori, 1985. Purification and characterization of an antibacterial peptide from haemolymph of *Sarcophaga peregrine* (fresh fly) larvae. J. Biochem., 211: 727-734.
13. Matsuyama, K. and S. Natori, 1988. Purification of three antibacterial proteins from the culture medium of NIH-Sape-4, an embryonic cell line of *Sarcophaga peregrine*. J. Biol. Chem., 263: 17112-17116.

14. Hara, S. and M. Yamakawa, 1995. Moricin, a novel type of antibacterial peptide isolated from the silkworm, *Bombyx mori*. J. Biol. Chem., 270: 29923-27.
15. Chowdhury, S., K. Taniai, S. Hara, S. Kadono-Okuda Y. Kato, M. Yamamoto, J. Xu, S. Choi, C. Nitish Debnath, Hong Kyu Choi, Akihiro Miyanoshita, Masao Sugiyama and Ai Asaoka Minoru Yamakawa, 1995. cDNA cloning and gene expression of lebocin, a novel member of antibacterial peptides from the silkworm, *Bombyx mori.* Biochemistry and Biophysics Research Communication, 214:271-278.
16. Taniai, K., K.Kadono-Okuda, Y. Kato, M. Yamamoto, M. Shimabukuro and S. Chowdhury, 1995. Structure of two S.cecropm B-encoding genes and bacteria-inducible DNA-binding proteins which bind to the 5 prime upstream regulatory region in the silkworm, *Bombyx mori*. Genetics, 163: 215-219.
17. Nakamura, T., H. Furanaka, T. Miyata, F. Muta, S. Iwanaga, M. Niwa, T. Takao and Y. Shimonishi, 1988. Tachyplesin, a class of anti-microbial peptide from the hemocytes of the horseshoe crab (*Tachypleus tridentatus*). J. Biol. Chem., 263: 16709-16713.
18. Morishima, I., K. Yamada and T. Ueno, 1992. Bacterial peptidoglycan as an elector of antibacterial protein synthesis in larvae of the silkworm *B. mori.* Insect Biochem. Molcular Biol., 22:363-367.
19. Gudmundsson, C.G., D.A. Lidholn, B. Asling, R. Gan and H.G. Boman, 1991. The cecropin locus: cloning and expression of a gene cluster encoding three antibacterial peptides in *Hyalophora cecropia.* J. Biol. Chem., 266: 11510-11517.
20. Rameshkumar, G. Isolation of antimicrobial peptide from the crab, *Thalamita crenata.* M.phil., Thesis, Annamalai University. pp: 41.
21. Veeruraj, A., S. Ravichandran and G. Rameshkumar, 2008. Antibacterial activity of crab haemolymph on clinical pathogens. Trends in Applied Sciences Research, 3(2): 174-181.