

HPTLC Separation Of Antibacterial Compounds From *Perna Viridis* And *Portunus Sanguinolentus* And Its Activity Tested Against Common Bacterial Pathogens

A.Maripandi^{1,2*} L. Prakash¹ Ali A. Al-Salamah²

Abstract

Marine invertebrates were identified as *Portunus sanguinolentus* (three spotted crab) and *Perna viridis* (Green mussel). Crude extract from both animals was tested against common laboratory pathogens *Escherichia coli*, *Klebsiella pneumonia* and *Staphylococcus aureus*. The result of *E.coli*, *K.pneumoniae* and *S. aureus* in ethanol and methanol extraction of *Perna viridis* was 4.88, 4.26, 5.74 and 5.33, 4.64, 4.97 and *Portunus sanguinolentus* 5.32, 4.66, 6.53 and 7.45, 5.23, 5.66 respectively. Further high performance thin layer chromatography separated 5 different compounds from both the animal extracts. Among compounds 3 and 5 more active against common laboratory pathogens. When compared to the crude extracts the purified component showed a high antibacterial activity against the tested pathogens. In addition to both active compounds from marine invertebrates were sensitive to proteinase K and heat treatment. Thus the active molecules are most likely of peptides. The compounds from the animals were tested with human RBC thus they possess no hemolytic activity. This study showed the possibility of utilizing marine invertebrates as new promising pharmaceutical therapeutics which could be used in pharmaceutical industries.

Key words: Marine invertebrates, HPTLC, Solvent, pathogens.

Introduction

The marine environment is a rich source of both biological and chemical diversity. About 80% of all life on earth is found under the ocean surface and two-thirds of the phyla are exclusively or dominantly marine. This diversity has been the source of unique chemical compounds with the potential for industrial development as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, fine chemicals and agrochemicals. In recent years, a significant number of novel metabolites with potent pharmacological properties have been discovered from the marine organisms. Although there are only a few marine derived products currently in the market, several new compounds from marine origin are now under clinical trials for drug development. While the marine world offers an extremely rich resource for novel compounds, it also represents a great challenge that requires multidisciplinary approach to bring the marine chemical diversity up to its therapeutic potential. Therefore, the marine environment, especially marine invertebrates that rely solely on innate immune mechanisms for host defense, is a spectacular resource for the development of new antimicrobial compounds. The recent appearance of a growing number of bacteria resistant to conventional antibiotics has become a serious medical problem. To overcome this resistance, the development of antibiotics with novel mechanisms of action is a pressing issue (Lohner *et al.*, 2001). The past three years has seen a revolution in the methods used to identify novel antimicrobial compounds from marine organisms.

Among the most promising are marine antimicrobial peptides. To date less than 5% of antimicrobial peptides have been identified (Douglas *et al.*, 2003; 2001). Antimicrobial peptides are wide spread in the living kingdom, and a large number of these molecules have been isolated from vertebrates and invertebrates. (Haug *et al.*, 2002). There is evidence that these molecules are largely found in marine invertebrates especially in tissues such as the gut and respiratory organs. (Chisholm *et al.*, 1992). 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. Cellular immunity in marine invertebrates is based on cell defense reactions, including encapsulation, nodule formation, and phagocytosis. The cellular component of marine invertebrate immunity is mediated by hemocytes, motile cells that phagocytize microbes and secrete soluble antimicrobial and cytotoxic substances into the hemolymph (Mitta *et al.*, 2000). More than 400 natural antimicrobial peptides and their modified synthetic analogues have been identified from a variety of invertebrates (Hancock *et al.*, 2000). These cationic antimicrobial peptides are genetically dissimilar to the conventional antibiotics and also these peptides have the ability to rapidly lyse the bacterial pathogen. Endogenous antimicrobial peptides are exciting candidates as new antibacterial agents due to their broad antimicrobial spectra, highly selective toxicities, and the difficulty for bacteria to develop resistance to these peptides (Hancock *et al.*, 2000). Their unique mode of action makes them as a new promise

1. PG and Research Department of Microbiology, K.S.Rangasamy College of Arts and Science, Periyar University, Tiruchengode – 637 209, Namakkal District, Tamil Nadu, India.

2. Unit of Medical Bacteriology, Department of Botany and Microbiology, College of Science, King Saud University, P.O.Box2455, Riyadh, Saudi Arabia. *Corresponding author. (Email address: drmari34@gmail.com)

pharmaceutical therapeutants. The present studies were *Portunus sanguinolentus* (Three spotted crab) and *Perna viridis* (Green mussel) extract by using methanol and ethanol and tested common laboratory pathogens.

Materials and Methods

Sampe collection

Live specimens of the *Portuna sanguinolentus* (Three spotted crab) and the *Perna viridis* (Green mussel) were obtained by trawling at the Puthiyappa Fishing Harbor, Calicut- West Cost of India and the Pervur Fishing Harbor, Tanjore – East Coast of India. The samples were individually packed in polypropylene bags and identified in Zoological Survey of India, Calicut.

Preparation of extract

Air dried samples were cut into small pieces and soaked in 95% Methanol and Ethanol (1g: 4ml) for one week. The crude extract was centrifuged and the centrifugate was concentrated under reduced pressure at 40°C using the evaporator. The concentrated methanol and ethanol extract was placed in a separator funnel and added with the equal amount of hexane solution. The mixture was shaken thoroughly to extract the non polar components from the crude extracts. Layers were allowed to separate and hexane layer was collected. The hexane layer was then subjected to evaporation under reduced pressure at 40°C. The residue was collected in vials and kept at freezer. The crude extracts were then subjected to extraction with chloroform to separate slightly polar components. Then the extracts were stored under freezer until the antimicrobial activity testing (Mojica *et al.*, 2000).

Bacterial strains and growth conditions

The gram-negative bacterial pathogens *Escherichia coli* and *Klebsiella pneumoniae* and the gram-positive pathogen *Staphylococcus aureus* were obtained from the Government Hospital at Erode.

Antimicrobial testing of crude extracts

Antimicrobial activity was evaluated using the agar diffusion technique in petri dishes (NCBL 2000). Briefly, 25µl of each extract was loaded on sterile filter paper discs 6mm in diameter and air-dried. Pathogenic microorganisms were spread on nutrient agar plates with sterile effusion and the discs were placed on plates. After incubation for 36- 48 hrs at 37°C, a clear zone around a disc was evidence of antibacterial activity. Diameter of the zones of inhibition were measured in millimeters each test was prepared in duplicate. Discs loaded with the extracting agent (Methanol and Ethanol) were tested as control.

High Performance Thin Layer Chromatography

The given Sample (given in paste form) 200mg was dissolved in 2ml methanol and filtered using whatman no.1 filter paper. This test solution contains 100µg / 1µl of extract concentration. 5µl of test solutions were applied on 5 x10 precoated silica gel 60 F₂₅₄ TLC plate (Merck) of uniform thickness of 0.2mm by using HPTLC-Linomat 5 system. The plate was kept in one troff and mobile phase in another troff of 10x10 twin troff chamber for plate saturation upto 10min. After saturation, the plate was developed in the respective mobile phase upto 8cm. Removed the plate and dried in hair drier. Viewed the developed plate in HPTLC-Reprostar-3 at Visible, UV254nm and UV366nm. Photo-documentation of TLC plate was also done. The respective Spray reagent was applied

over the plate and heated at 110°C for 5 min. Immediated the plate was Photo-documented using the HPTLC-Reprostar 3 at Visible, Uv254nm and UV366nm. The plate was scanned at 254nm using HPTLC-TLC Scanner 3 and the spectrum was taken.

Proteinase k and heat treatment

Fractions showing antibacterial activity were tested for proteinase k sensitivity using the standard procedure (Haug *et al.*, 2001). Proteinase k was dissolved in 50mM Tris HCl (pH 7.5) at a concentration of 2.5mg/ml. The fractions was diluted in Milli-Q water to a protein concentration of 250µg/ml. A volume of 10µl proteinase K solution was added per 50µl sample. The mixture was incubated at 42°C for 90min for protein digestion. The temperature was then elevated to 85°C for 15 min to inactivate the proteinase K. As a control (Heat treatment) 10µl 50Mm Tris-HCl (pH 7.5) was added to 50µl of the diluted sample and subjected to the same treatment as the proteinase K sample. The activities in the treated samples were determined in microtitre plates as described above. Fractions that no longer showed antibacterial activity after proteinase K/heat treatment were regarded as sensitive.

Hemolytic Assay

The hemolytic assay of the active components was performed using the standard method (Haug *et al.*, 2001). To test whether the animals contain factors which are toxic to eukaryotic cells, the hemolytic activity in extracts from whole body were determined using fresh human red blood cells (RBC). 4 ml of blood were collected from a healthy person into a polycarbonate tubes containing heparin to a final concentration of 10U/ml. The erythrocytes were isolated by centrifugation at 450g for 10 min and washed three times with phosphate buffered saline (pH 7.4) in order to remove plasma and buffy coat. The cell pellet was resuspended in 4ml of PBS. The test samples were diluted to a protein concentration of 500µg/ml and the test was performed in 96 well U shaped microtitre plates. To each well was added 40µl PBS, then 50µl test fraction and lastly 10µl of the RBC suspension. After incubation in a shaker at 37°C for 1h the plates were centrifuged at 200g for 5 min. Base line haemolysis and 100% haemolysis were defined as the amount of hemoglobin released in the presences of PBS and 0.1% Triton X -100 (Sigma), respectively.

Results

The Collected Samples were identified by the Experts in the Calicut Centre of Marine Fisheries Research Institute (CMFRI) and confirmed at Zoological survey of India at Calicut. They are *Portunus sanguinolentus* (Three spotted crab) and *Perna viridis* (Green mussel)

Antimicrobial testing of crude extracts

The ethanol and methanol extract of *Perna viridis* and *Portunus sanguinolentus* were tested against *E.coli*, *Klebsiella pneumonia* and *Staphylococcus aureus*. The extract of *Perna viridis* and *Portunus sanguinolentus* antibacterial activity results (Table 1; Fig 1 a&b)

High performance thin layer chromatography purification:

The components present in both the extracts were separated by high performance thin tlayer chromatography (HPTLC). There are four different components separated at different RF values from both the extracts. These components are scanned at 254 nm through the HPTLC scanner and measure the peak value of the components. Lamda max

<i>Perna viridis</i> and <i>Portunus sanguinolentus</i> antibacterial activity of common bacterial pathogens				
Marine organisms	Pathogens used for testing extract	Strength of crude extract	Zone of inhibition(mm)	
			Methanol	Ethanol
<i>Perna viridis</i>	<i>E.coli</i>	25µl	5.32	4.88
	<i>Klebsiella pneumonia</i>		4.66	4.26
	<i>Staphylococcus aureus</i>		6.53	5.74
<i>Portunus sanguinolentus</i>	<i>E.coli</i>	25µl	7.45	5.33
	<i>Klebsiella pneumonia</i>		5.23	4.64
	<i>Staphylococcus aureus</i>		5.66	4.97

Table 1.

*Zone of inhibition of marine animal extracts mean value of triplicate test

analysis of the component 1-5 of both the extracts shows that the same spectrum at 247nm. It indicates that the same component is present in both extracts but the amount which could vary (Fig2). The separated compound from the each extracts was tested against the pathogenic

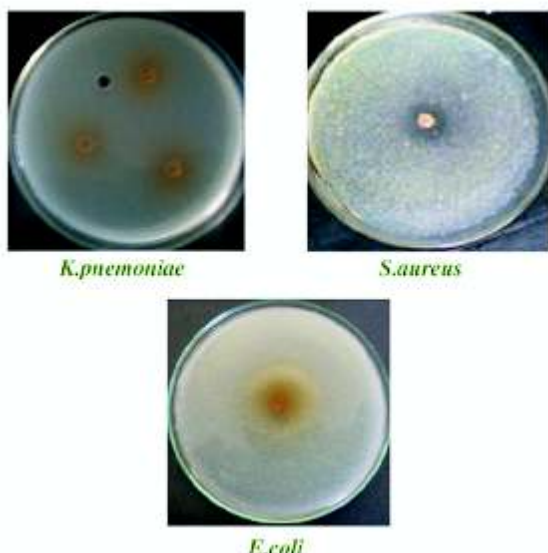


Fig 1 a. Antibacterial activities of crude extract perna viridis

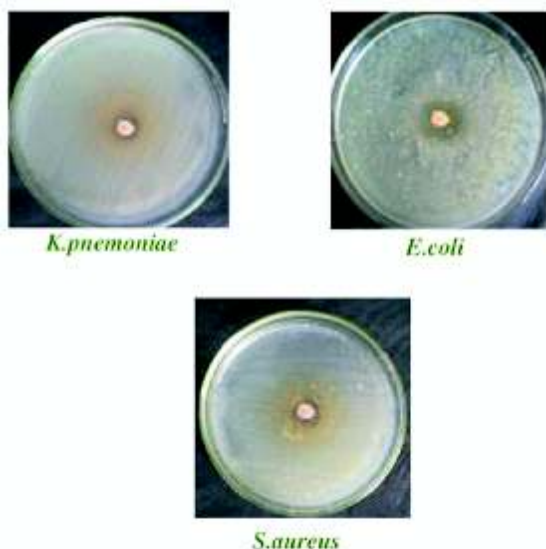


Fig1 b. Antibacterial activities of crude extract *Portunus sanguinolentus*

HPTLC separation of <i>Perna viridis</i> and <i>Portunus sanguinolentus</i> antibacterial compounds and its activity tested against common bacterial pathogens						
Marine organisms	Pathogens used for testing extract	Strength of purified extract	Zone of inhibition(mm)			
			Methanol		Ethanol	
			C3	C5	C3	C5
<i>Perna viridis</i>	<i>E.coli</i>	25µl	28.43	4.34	26.12	4.54
	<i>Klebsiella pneumonia</i>		26.52	4.65	24.34	5.21
	<i>Staphylococcus aureus</i>		28.22	5.12	27.44	5.33
<i>Portunus sanguinolentus</i>	<i>E.coli</i>	25µl	26.21	3.00	23.11	3.21
	<i>Klebsiella pneumonia</i>		14.54	3.22	10.56	3.11
	<i>Staphylococcus aureus</i>		25.33	5.13	21.67	4.54

Table 2

*Zone of inhibition of marine animal extracted compounds^o mean value triplicate test

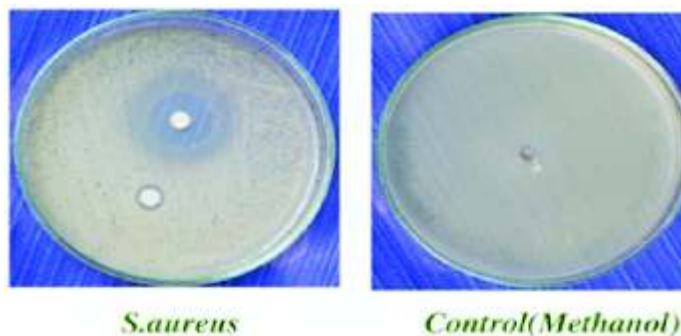


Fig .2 a. Antibacterial activity of perna viridis extracted compounds 3 and 5.

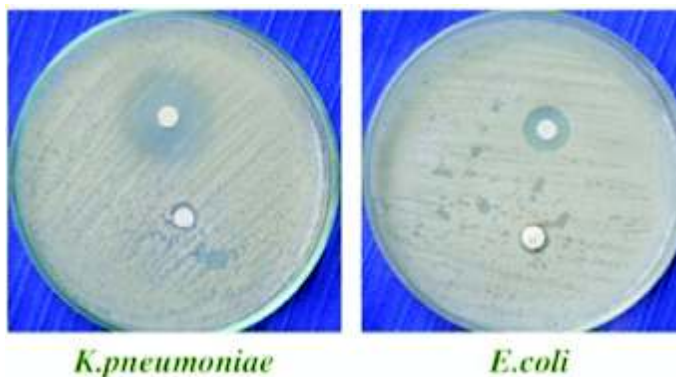


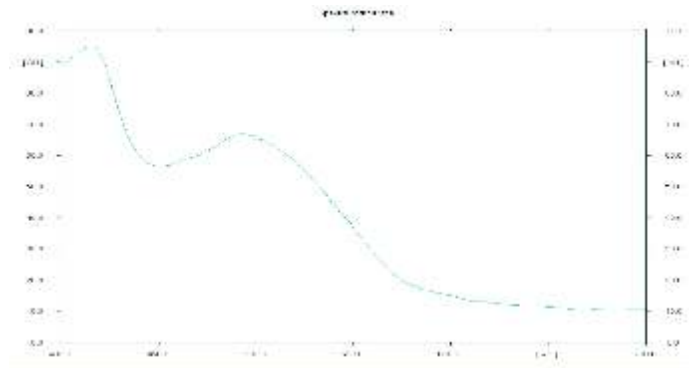
Fig 2 b. Antibacterial activity of *Portunus sanguinolentus* extracted compounds 3 and 5



Fig.2. HPTLC analysis of *Perna viridis* (1) and *Portunus sanguinolentus*(2)

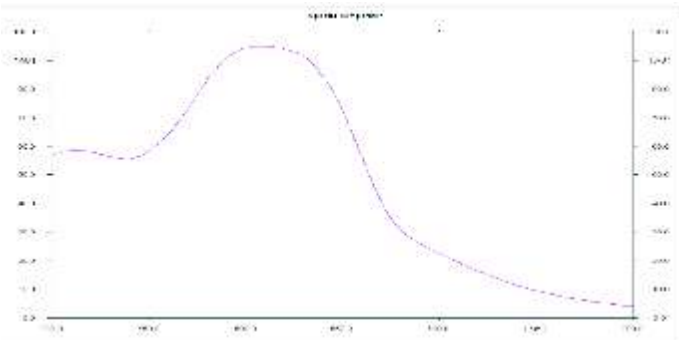
HPTLC analysis- Peak value of the separated component at 254nm after derivatization

S.no.	Track	Peak	Rf	Height	Area
1	1	1	0.27	71.2	3083.1
2	1	2	-	-	-
3	1	3	0.35	137.2	4250.4
4		4	0.43	182.9	8177.3
5		5	0.53	166.2	8630.1
6	1	1	0.59	130.8	3524.9
7	2	1	-	-	-
8	2	2	0.26	327.1	14751.9
9	2	3	0.33	232.1	9499.4
10	2	4	0.41	258	14296.5

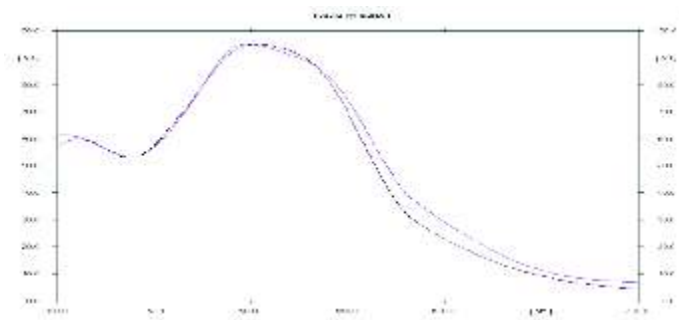


max of peak-1 is measured at 415nm
Dark green line – Peak 1 of Crab

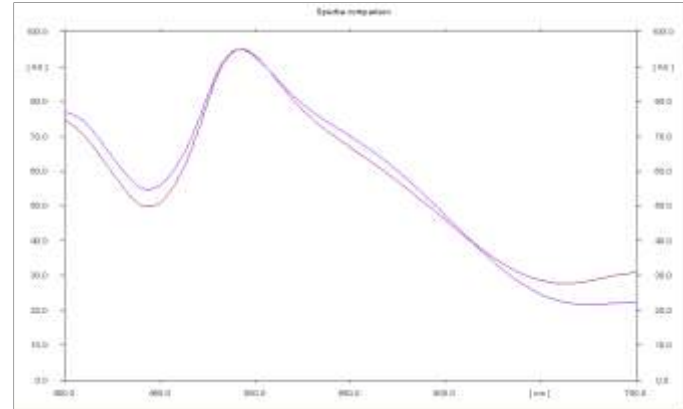
Spectrum of peak-2 at R_f – 0.26
max of peak-2 is measured at 507nm



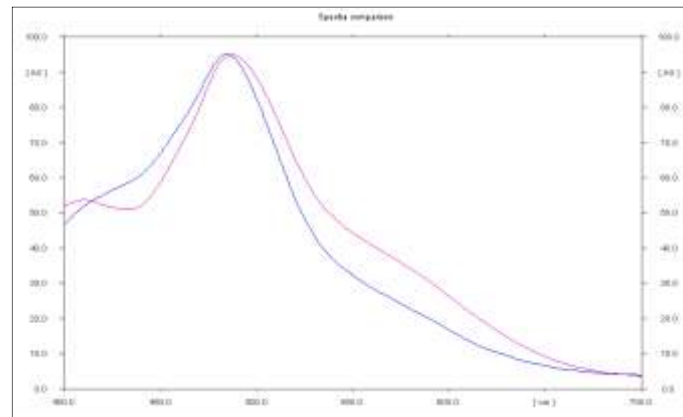
Pink line Peak 2 of Green mussel
Spectrum of peak-3 at R_f 0.32
max of peak-3 is measured at 503nm.



Dark brown Peak 3 of Crab; Violet Peak 3 of Green mussel
Spectrum of peak-4 at R_f 0.41
max of peak-4 is measured at 492nm



Dark brown – Peak 4 of Crab; Dark violet – Peak 4 of Green mussel
Spectrum of peak-5 at R_f – 0.50
max of peak-5 is measured at 487nm.
Pink – Peak 5 of Crab; Blue – Peak 5 of Green mussel



microorganisms. From these results, we observed that the component 3 and 5 present in both the extracts is responsible for antibacterial property.

Proteinase K sensitivity

Active fractions from *Perna viridis* and *Portunus sanguinolentus* have sensitive to proteinase K thus the results indicate that the fractions were protein in nature and no activity detected against common laboratory pathogens.

Hemolytic assay

There was no hemolytic activity detected from active components from *Perna viridis* and *Portunus sanguinolentus* (Fig 3).



Fig 3 Hemolytic assay of *Perna viridis* and *Portunus sanguinolentus*

Discussion

A screening for antibacterial activity in whole body of marine invertebrate *Portunus sanguinolentus* (Three spotted crab) and *Perna viridis* (Green mussel) was conducted. The results showed that the two species possess antibacterial activity against common laboratory pathogens tested *in vitro* condition. Antibacterial activity has been previously described in a wide range of crustaceans (Haug *et al.*, 2002) and mollusca species (Mitta *et al.*, 2000). In most of the species studied the whole body or body wall has been tested for activity. The present study demonstrates the presence of antibacterial factors might therefore have an important function as a first line of defense against pathogenic bacteria. The crude extract of both these extracts showed antibacterial activity against *K. pneumoniae*, *E.coli*, and *S. aureus*. The partial purification using high performance thin layer chromatography method separated compounds according to their hydrophobicity. The separated compound screened for antibacterial activity the compounds 3 and 5 (*Portunus sanguinolentus*) and 3 and 5 (*Perna viridis*) was responsible for antibacterial activity. Among these compounds, the compound 4 from both the extracts showed high antibacterial activity. When compared to the crude extracts the purified component showed a high antibacterial activity against the tested organisms. In addition the active compounds from both the extracts were sensitive to proteinase K and heat treatment. These results indicate that the marine crustaceans and mollusk have developed a variety of defense molecules against pathogenic microorganisms. Since enzymatic digestion reduces the antibacterial activity and thus the results indicate that the active compounds are mostly protein nature. Antimicrobial peptides and proteins have previously been described identified in crustaceans (Destoumiex *et al.*, 1997; 2000) and mollusk. When the proteinase K treatment was performed, the heat treatment was included in the test to ensure that the antibacterial activity detected was not caused by proteinase K itself. The heat labile components might therefore also be sensitive to proteinase K treatment and contains proteins and peptides with antibacterial activity. Both the active components showed no hemolytic activity against human red blood cells. From a pharmaceutical point of view, it was an advantage when antibacterial drugs have no side effects, such as hemolytic activity.

Acknowledgement

The authors are thankful to the Principal and the Management, K.S.R. College of Educational Trust, Tiruchengode, Tamilnadu, India, for providing necessary facilities and constant encouragement to carry out

this study and also thanks to Dalmia Centre for Research and Development who was providing HPTLC analysis of marine samples at Coimbatore, Tamilnadu, India.

Reference

- Chisholm, J.R.S, and Smith, V.J. 1992. Antibacterial activity in the hemocytes of the shore crab *Carcinus maenas*. *J.Mar.Biol.Assoc.***72**: 529-542.
- Destoumiex, D, Bulet, P, Loew, D, Dorsslaer, A.V. 1997. A new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (decapods). *J. of biolo chem.* **272**: 28398-28406,
- Destoumiex, D., M. Munoz, P. Bulet, and E. Bachere. 2000. Penaeidins, a family of antimicrobial peptides from penaeid shrimp. *Cell. Mol. Life Sci.* **57**:1260-1271
- Douglas, E, Aleksander P, Jennifer P, and Jeffrey W.Gallant 2003. Identification, Structure and differential expression of novel Pleurocidins clustered on the genome of the Winter flounder, *Pseudopleuronectes americanus* (Walbaum). *Eur. J. Biochem.* **270**: 3720-3730.
- Douglas S, J.W. Gallant, Z.Gong, C.Hew 2001. Cloning and developmental expression of a family of Pleurocidin- like antimicrobial peptides from Winter flounder, *Pleuronectes americanus* (Walbaum). *J. Develop and Comparative Immunol* **25**: 137-147.
- Mojica, E, R.J.Layson, A.Rodil, C.Deocariz. Marine invertebrates as source of potential Anti-tumor and Antibacterial agents.
- Hancock, R.E., Rozek, A., 2000. Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbial. Lett.***206**, 143-149.
- Lohner, K., and E.Staudegger 2001. Development of novel antimicrobial agents: emerging strategies. Horizon scientific press, Wymondham, United Kingdom.
- Mitta, G., F.Hubert, E.Dyrynda, P.Boundry, and P.Roch, 2000. Myticin B and MGD 2, two antimicrobial peptides of marine mussels: gene structure and expression analysis. *Dev. Comp. Immunol.***24**:381-393.
- Haug T., K.Anita .Kjuul,Klara Stensvag, E.Sandsdalen and B.Styrvold (2002). Antibacterial activity in four marine crustacean decapods. *J. Fish and shellfish Immunology* **12**: 371-385.