

Antimicrobial lipids from the hemolymph of brachyuran crabs

S. Ravichandran^{*1}, Solimabi Wahidulla², Lisette D'Souza² and G. Rameshkumar¹

¹Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai – 608 502, India

²National Institute of Oceanography, Council of Industrial and Scientific Research,
Dona Paula- 403 004, Goa, India

Phone: +91-832- 2450 450, FAX: + 91 0832-2450 602 /03

Corresponding author*

Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai 608502, INDIA

Phone: + 91 - 4144 - 243223, 243533, FAX: + 91 - 4144 — 243555, [E-mail: sravicas@gmail.com](mailto:sravicas@gmail.com)

Abstract

The potential of marine crabs as a source of biologically active products is largely unexplored. In the present study antimicrobial activity of the hemolymph (plasma) and hemocytes (plasma cells) of six brachyuran crabs was investigated against 16 pathogenic strains. Among the 16 strains tested maximum zone of inhibition was recorded in the hemolymph of *Hyas araneus* against *Shigella flexineri*. Interestingly *Staphylococcus aureus* and *Salmonella typhi* were susceptible to all the hemolymph and hemocytes samples. Likewise, highest zone of inhibition was exhibited by both hemolymph and hemocytes samples against *Vibrio cholera*. On the basis of TLC, ¹HNMR and ¹³CNMR it may be concluded that the antimicrobial activity in the hemolymph extract is due to the presence of lipids. This observation is further supported by the ESI-MS of the methanolic extract of hemolymph of *Hayas araneus*. ESI-MS shows cluster of peaks in the region m/z 445 to m/z 491 due to lysoglycerolipids/glycerides and cluster of signals between m/z 216 to 246, due to fatty acids/esters present in the sample.

Key words: Crab, Hemolymph, Lipid, Antimicrobial, TLC, ESI-MS

Abbreviations

TLC : Thin Layer Chromatography

^1H NMR : Proton Nuclear Magnetic Resonance ^{13}C NMR : Carbon 13 Nuclear Magnetic Resonance m/z
: mass-to-charge

ESI-MS : Electrospray Ionisation Mass Spectrometry

mm : millimeter

cm :centimeter

MS/MS : Tandem Mass Spectrometry

NaCl : Sodium Chloride

pH : Hydrogen Ion Concentration

w/v : weight/volume

V/V : volume of the substance

l : microliter

g : microgram

FTIR : Fourier Transform Infrared Spectroscopy

MeOH : methanol

H₂O : water

TFA : Trifluoro Acetic Acid

CID : Collision-induced Dissociation

TOF : Time-of-flight

V : Volt

PDB : Potato dextrose broth

SDB :Sabaraud dextrose broth

Introduction

The field of marine natural products has been expanding in response to the growing number of structurally novel and biomedically promising natural products being isolated from the marine source. Marine invertebrates rely solely on innate immune mechanisms that include both humoral and cellular responses. The circulating hemolymph in marine invertebrates contains biologically active substances such as complement, lectins, clotting factors, antimicrobial peptides and lipids such as fatty alcohols, free fatty acids and monoglycerides [1, 2]. A clinical use of antimicrobial lipids was suggested many years ago by several authors [3-5], who emphasized the relative lack of toxicity of antimicrobial lipids from natural sources, and for many years undecylenic acid has been used in topical over-the-counter antifungal preparations [6]. However, no pharmaceutical products containing lipids as active compounds have as yet been approved for clinical use as prophylactic or therapeutic drugs against bacterial infections.

A lectin from the *Scylla serrata* crab hemolymph has been isolated and purified by affinity column chromatography and preparative electrophoresis [7]. Based on studies of the health-promoting activities of lipids, both *in vitro* and *in vivo*, the possibility of using such lipids as active ingredients in prophylactic and therapeutic dosage is considered [8,1].

Some of the brachyuran crabs have shown pronounced activities, useful in the biomedical area. 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 [9-11]. The potential of marine crabs as a source of biologically active products is largely unexplored. Hence, a broad, based screening of marine crabs for bioactive compounds is necessary. Literature survey revealed that little is known about the antimicrobial compounds from marine brachyuran crabs, hence the present study was undertaken to investigate antimicrobial potency of these crabs.

Materials and Methods

Collection of crab Hemolymph

Brachyuran crabs were collected from neritic and oyster zone environment of Vellar estuary (Lat 11° 29'N; 79°46'E). Healthy male and female crabs at different stages of development were used for

experimental purpose. Hemolymphs were collected by cutting each leg of the live animal with a fine sterile scissor. To avoid hemocyte degranulation and coagulation, the hemolymph was collected in sodium citrate buffer, pH 4.6 (2:1, V/V) to which equal volume of physiological saline (0.85%, NaCl, w/v) was added. This was followed by centrifugation at 2000rpm for 15 min at 4°C to remove hemocytes from the hemolymph. Supernatant was collected by aspirating, stored at 4°C and tested within 16 hours.

Microbial strains used and Growth conditions

Antimicrobial activity of methanolic extracts of crabs hemolymph and hemocytes samples was evaluated against 16 different bacterial, fungal and multi drug resistant bacterial strains viz, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella flexneri*, *Klebsiella* sp., *Vibrio cholerae*, *Aspergillus fumigatus*, *Rhodotorula* sp., *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus niger*, *Streptococcus pyogenes*, *Acinetobacter* sp., *Salmonella typhi* and Methicillin Resistant *S. aureus*.

Cultures were maintained on Nutrient Agar slants at 4°C and regularly subcultured every month. Subculturing of fungi was done from broth to broth using either PDB (potato dextrose broth) or SDB (Sabaraud dextrose broth) media. The clinical isolates were grown overnight in Nutrient Broth before evaluating antimicrobial activity of the crude extracts.

Determination of antimicrobial activity

The susceptibilities to antimicrobials were determined according to the technique of Bauer et al [12]. Antibacterial and antifungal activity was expressed in terms of radii of zone of inhibition and was measured in mm using a scale.

Antimicrobial Assay

Clinical isolates were spread plated (200 l) onto Mueller Hinton Agar no 2 plates using a sterile spreader, taking care to distribute the microorganisms as evenly as possible over the agar surface.

Hemolymph and hemocytes samples were taken in methanol and the extracts were prepared for the assay in the following manner. Sterile discs (6 mm diameter) made of Whatman No1 filter paper, or prepunched (S and S Sterile blanks) were systematically laid out on a clean aluminum foil in such a

manner that each extract was assayed in triplicate against each microorganism. The discs were loaded each with (10) L of the extract by first applying 5 L with the pipette, allowed to evaporate, then applying another 5 L, then drying again. The discs were then placed individually using a sterile forceps in appropriate grids which were marked on undersurface of the plated petri plates and kept for incubation at room temperature (27°C) for 24 hrs. After incubation, plates were observed for zones of inhibition and recorded in millimeters.

Negative controls were methanol, chloroform and water. Positive controls are provided in ready-to-use standard discs: Penicillin G (Himedia), 10 g/disc; ketoconazole (Himedia) 10 ug/disc. Four (4) discs were placed in each agar test plate (in some instances as much as 7) by using a sterile forcep and then pressing them on the agar very lightly. The plates were incubated overnight at 37°C after which the zones of inhibition were measured in mm using a scale.

Thin layer chromatography (TLC)

A diluted methanolic solution of the extracts were applied to the thin layer chromatographic plate (Merck) with a capillary tube and placed in a chamber containing methanol:chloroform (5:95) as developing solvent. After development compounds were visualized as purplish pink spots on spraying with 5% methanolic sulphuric acid as detecting agent followed by heating at 100° C till the spots were visible. Heating for longer period turn the spots black.

General experimental procedures

Infra-red spectrum was recorded on Shimadzu FTIR 8201 PC spectrometers. NMR (¹H, ¹³C) data were obtained on Bruker Avance 300 spectrometer with TMS as internal standard. EIMS and MS/MS were recorded on QSTARXL MS/MS, applied Biosystems, Switzerland.

ESI-QTOFXL MS/MS spectrometry (Tandem mass spectrometry)

ESI-MS and MS/MS spectra were recorded, in the positive ion mode, on a QSTARXL MS/MS applied Biosystem instrument (Canada) equipped with Analyst Software. ESI-MS (electrospray ionization mass spectrum) of methanolic extract of hemolymph samples was recorded by dissolving the sample in MeOH: H₂O containing trace of 0.1% TFA. The solution was directly infused at the constant flow rate of 10l/min in to the ion spary source using integrated syringe pump. The declustering potential

and the collision energy (25-40V) were optimized for MS/MS experiments so as to cause fragmentation of the selected molecular ion species as evident by the appearance of the fragment ions and decrease in the intensity of the molecular ion.

Results

Antimicrobial Assay

Antimicrobial activity of the methanolic extract of hemolymph (plasma) and hemocytes (plasma cells) of six brachyuran crabs (*Hyas araneus*, *Podophthalmus vigil*, *Dromia dehanni*, *Charybdis helleri*, *Portunus sanguinolentus*, *P. pelagicus*) against 16 bacterial and fungal pathogenic strains is summarized in Table 1. It is evident from the results that activity is observed in all the species of crabs tested. Among the 16 strains tested maximum zone of inhibition (6 mm) was recorded in the hemolymph of *Hyas araneus* against *Shigella flexineri*. Interestingly *S. aureus*, *Salmonella typhi* were susceptible to all the hemolymph and hemocytes samples; the latter being more sensitive to hemocytes of *Dromia dehanni*. *Vibrio cholera* seemed to be the most sensitive microorganism to crude methanolic extracts of hemolymph as well as hemocytes samples of all the six brachuran crabs except hemolymph of *P. pelagicus* of the present investigation.

The extracts were ineffective against the bacteria *Klebsiella* sp and Multi-drug resistant *Salmonella typhi* and fungal strains, *Rhodotorula* sp., *Candida albicans*, and *Aspergillus niger*. The tested methanolic extracts of hemolymph and hemocytes samples exhibited very good activity against bacterial strains as compared to multi-drug resistant bacteria and fungal strains.

H. araneus looked promising in terms of antimicrobial activity; this observation led us to investigate this organism in detail. The infra-red spectrum of the crude methanolic extract (Fig. 1) was typical of a polyhydroxy compound with a broad band between 3100- 3600 cm^{-1} and 1010 cm^{-1} due to the hydroxyl functionality. A pronounced signal at 1750 cm^{-1} was assigned to the carbonyl group of the ester. Unsaturation is evident by the presence of signals at 3030 cm^{-1} and the broad absorption centered at ~1650. The peaks at 2925 and 2854 cm^{-1} for aliphatic CH stretching were also present in the spectrum.

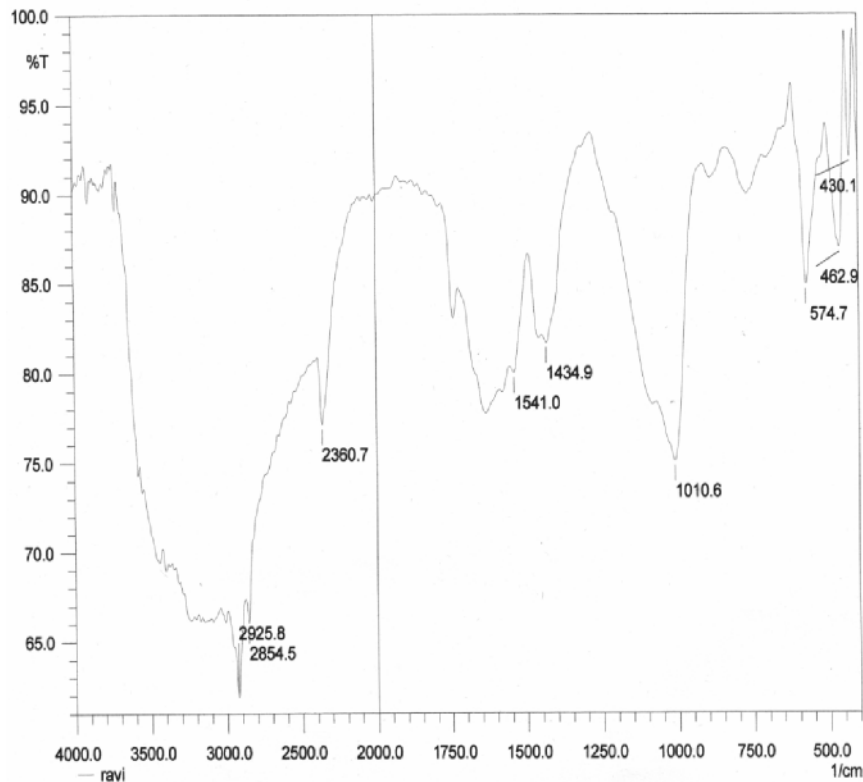


Figure 1. Infrared spectrum of the methanolic extract of the crab, *H. araneus*

The ^1H NMR spectrum (Fig.2) was typical of lipids with special reference to fatty acids and glycerolipids. It exhibited two terminal methyl signals, as triplet at 0.874 (3H, t, $J= 6.9\text{Hz}$) due to the presence of saturated fatty acyl chain and a terminal methyl residue at 0.9 15 (3H, t, $J= 6.9\text{ Hz}$) of unsaturated fatty acyl chain; a broad signal at 1.274 was assigned to the methylene protons of the aliphatic chains of the two acyl groups; multiplets centered at 2.33 8, 2.07 9 and 1.643 were attributed to the methylene groups linked , , and respectively to the ester/acid functional group; a triplet at 2.86 is usually observed for the allylic methylene group of the type present in unsaturated fatty acyl group such as linolenic acid. The olefinic methane protons were evident at 5.38. The sugar protons were apparent in the region 3.71-4.36 but seem to occur in minute quantities as compared to other fatty matter. The methyl of rhamnosyl sugar moiety usually occurs as a doublet in the ^1H NMR and though there is overlapping, this is evident in the present spectrum, with a signal centered at 1.00 (d, $J=7.2\text{ Hz}$) and the corresponding absorption in ^{13}C NMR at 17.7ppm.

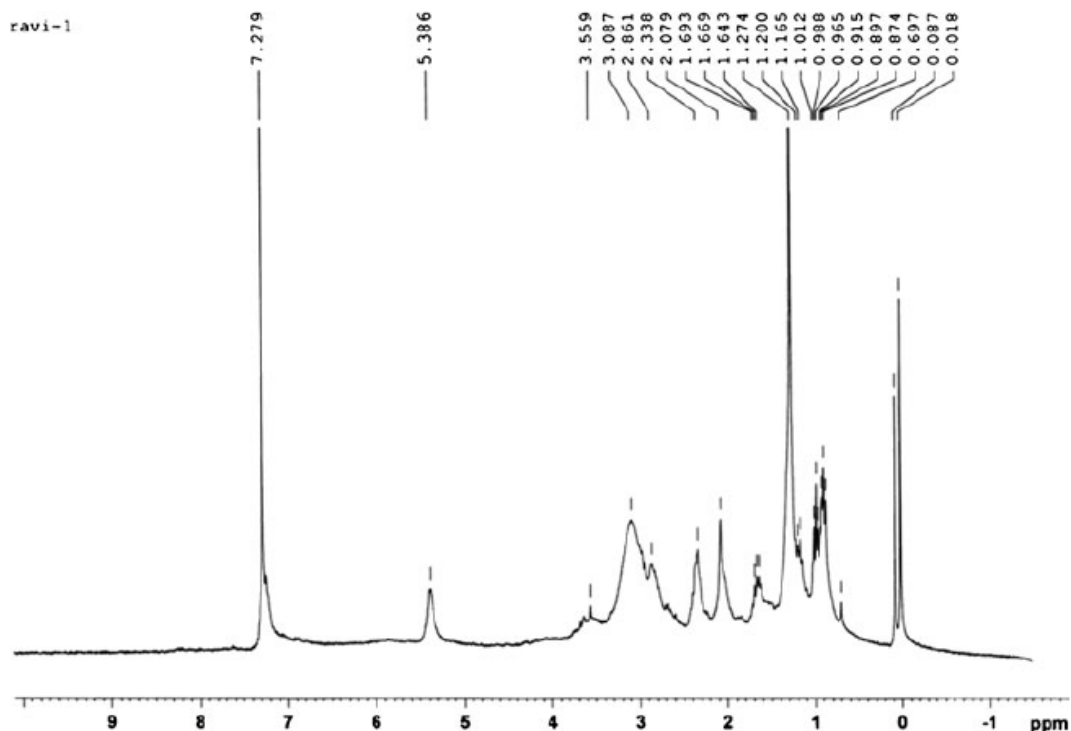


Figure 2. ¹H NMR spectrum (CDCl₃) of the methanolic extract of hemolymph of *H. araneus*

The ¹³C NMR spectrum of the sample (Figs 3) showed two quarternary carbons at 172.5, 173.8, and additional two at 178.1 and 180.2. Former two for ester carbonyl functions of glycerides/glycerolipids/fatty acid esters and the latter two for the fatty acids present in the mixture; terminal methyls of the fatty chain were evident in the region 14.06-20.5, methylene carbons were evident in the region between 20.5 and 34.22. The unsaturation of the aliphatic chain was evident between 125.3- 129.32 with the most intense signals in this region being present at 127.0, 128.4, 128.9 and 129.3. This indicates that the glycolipids contain acyl group wherein the fatty acids present belong to C18 group with either one as in oleic acid (18:1) or two double bonds as in linoleic acid (18:2). It is also indicative of presence of glycerides evidenced by the presence of ¹³C NMR signals due to glycerol resonance at 59.4, 60.2 and 68.3 ppm. Presence of saturated ketone is indicated by the presence of signals at 202.8 and 209.0 in the ¹³C NMR of the sample.

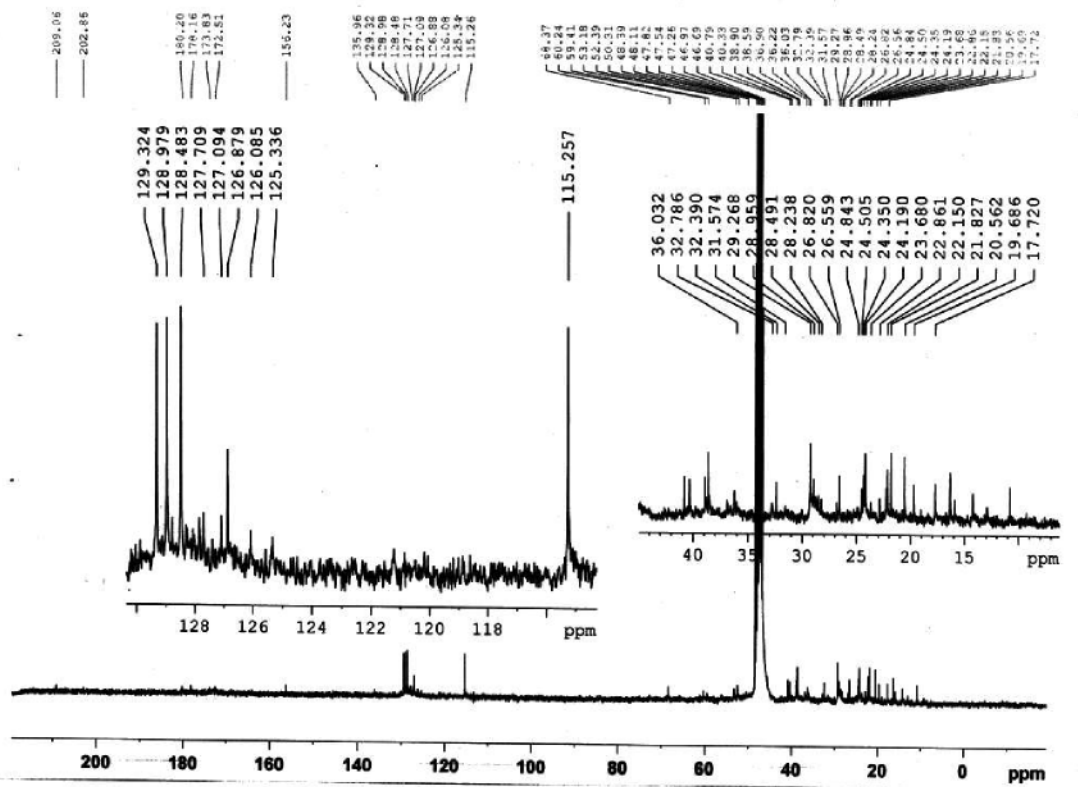


Figure 3. ^{13}C NMR spectrum (CD_3OD) of the methanolic extract of hemolymph of *H. araneus*

On the basis of foregoing evidence it is concluded that the antimicrobial activity observed in the extract is due to the presence of lipids. This observation is further supported by the ESIMS (Fig. 4) profile of the methanolic extract of haemolymph of crab *H. araneus*. ESI-MS shows cluster of peaks in the region m/z 445 to m/z 491 due to lysoglycerolipids and another cluster in the region of m/z 216 to m/z 246 probably due to the presence of fatty acids in the sample. From ESI-MS spectrum molecular species with molecular mass of 474 amu and 460 were subjected to tandem mass analysis

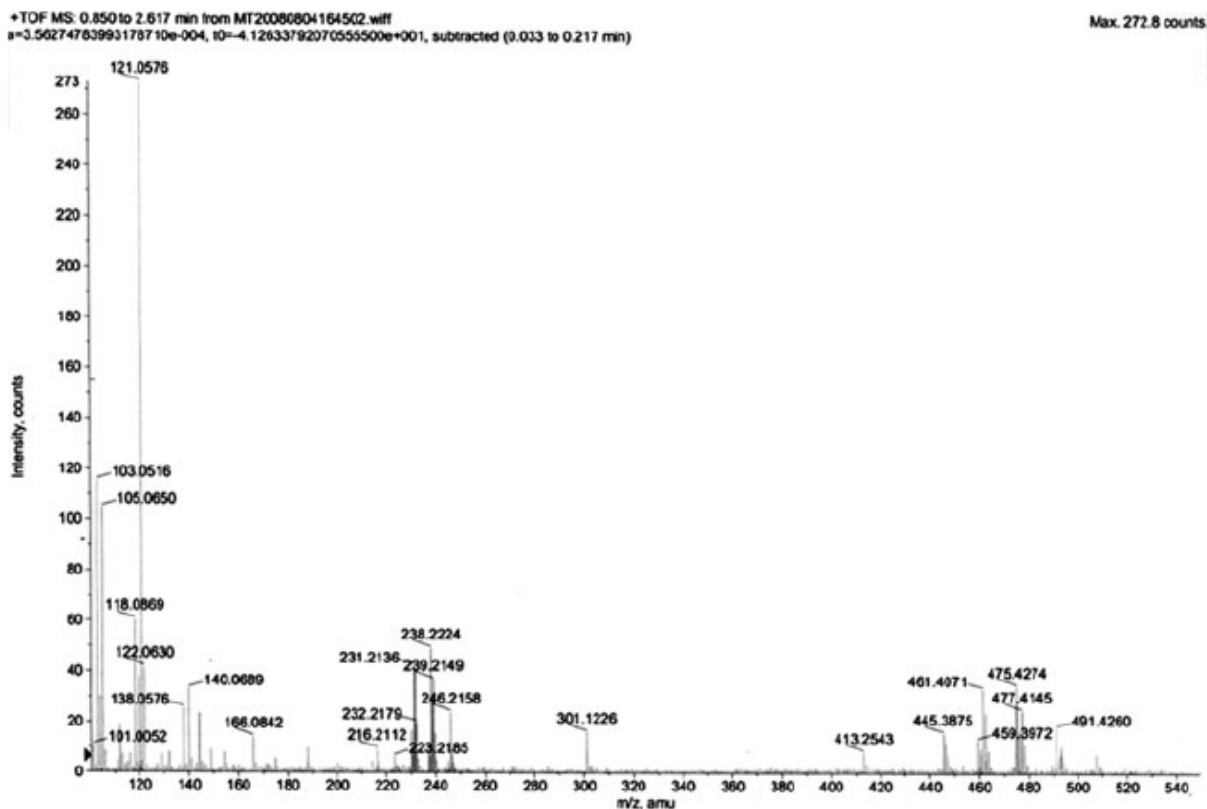


Figure 4. Positive ESI-MS of the infused extract of hemolymph of *H. araneus*

Tandem mass spectra (Fig. 5 and Fig 6) of the molecular species with $[M+H]^+$ at m/z 461 and m/z 475 indicated that the lipid contains palmitate acyl group evidenced by the presence in both the spectra, of the signals due to loss of palmitate acyl group ($C_{15}H_{31}-C=O$) with atomic mass of 239 amu (signal at m/z m/z 222 in the former and at m/z 236 in the latter) and also due to the loss of palmitic acid. Since the cleavage at sn-2 position is preferred based on the intensity of the signal the palmitate group was placed at position C-2 of the glycerol moiety [13,14]. The signal at m/z 164 was attributed to protonated hexose moiety probably rhamnosyl and not galactosyl/glucosyl as it is usually expected because the mass of m/z 475 is in agreement with the presence of deoxy sugar. On the basis of above data the glycolipid with m/z 475 has been identified as 3O-rhamnosyl-2-palmitoyl glycerol and assigned structure as shown in the Fig. 7.

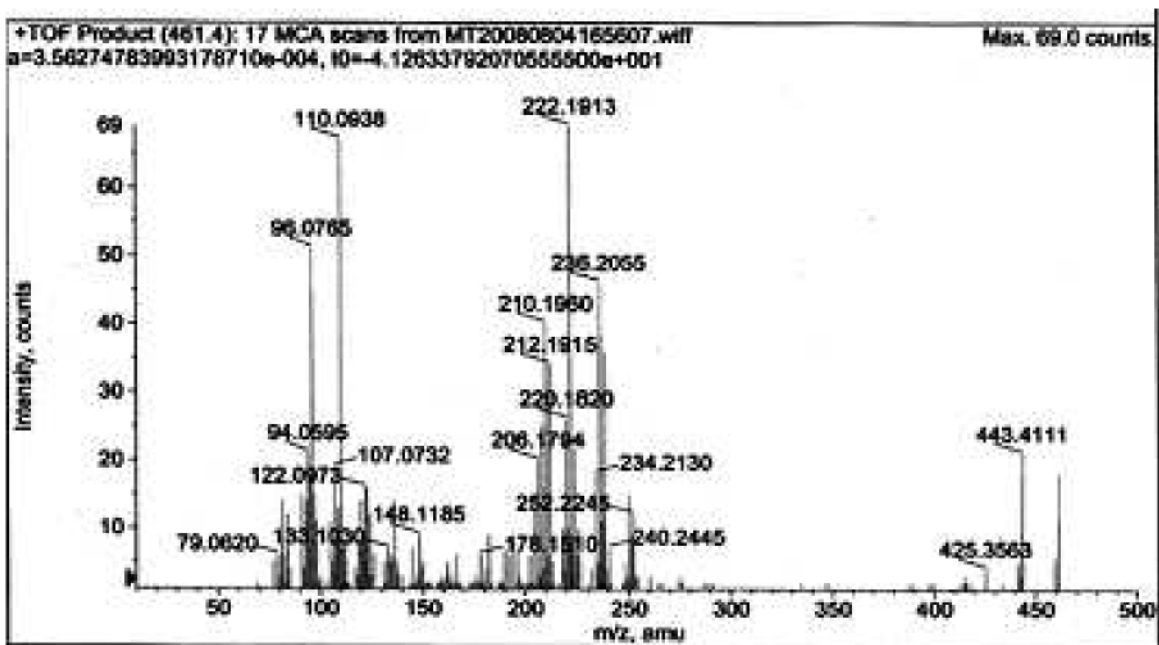


Figure 5. Positive ESI-MS/MS of the molecular species with $[M+H]^+$ at m/z 461

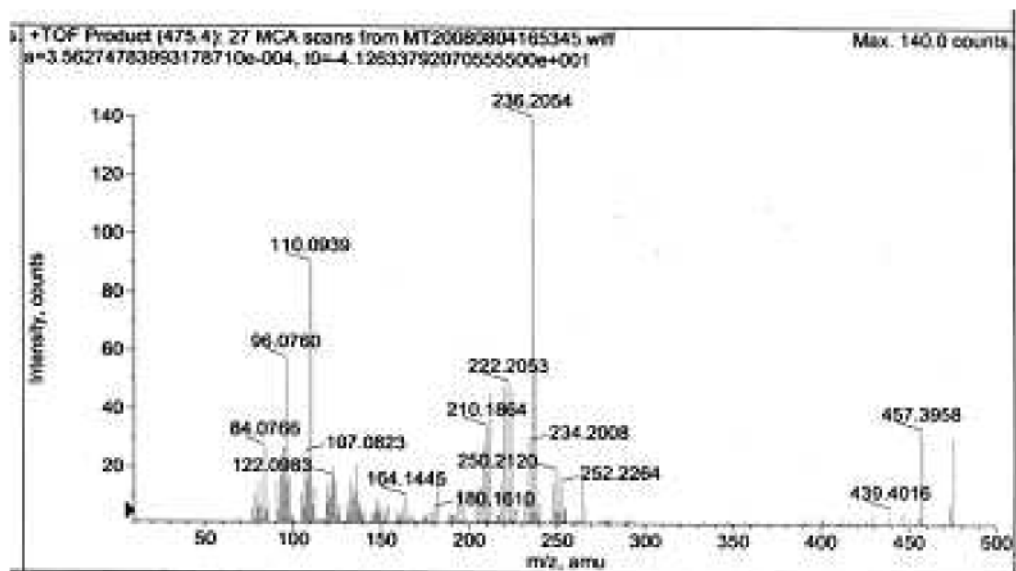


Figure 6. Positive ESI-MS/MS of the molecular species with $[M+H]^+$ at m/z 475

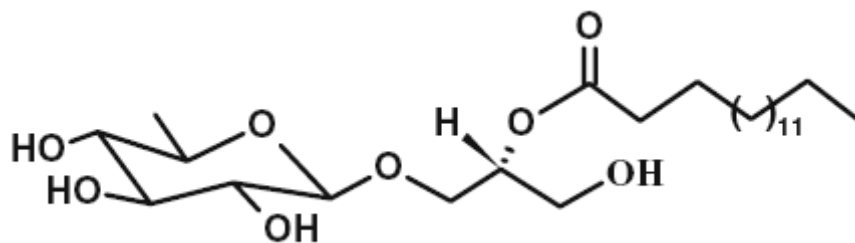


Figure 7. Structure of the glycolipid with molecular mass of 475amu.

In the ESI-MS of methanolic extract of haemolymph of *H. araneus* as the molecular mass of the two molecular species, m/z 475 and m/z 491 differ by 16 amu (Fig. 5), it is indicative of the extract also containing lysoglycerolipid with glucose/galactose as the sugar moiety. The molecular species with molecular mass of m/z 460 has been identified as 3O-galactosyl/ glucosyl-2-palmitoyl glycerol. Galactosyl glycolipids are very common.

Discussion

The emergence of new infectious diseases and resistance to the antibiotics by the existing ones led to the new sources for drug discovery. Many organisms possess antimicrobial properties, although most of the antibacterial agents that have been isolated from marine sources have not been active enough to compete with conventional antimicrobials obtained from micro organisms [15-17]. However, the research on marine organisms is still in its infancy considering the vast resources that still remains untapped with majority of marine organisms yet to be screened for the discovery of useful antibiotics.

In the present study, the methanolic extract of crab hemolymph showed antimicrobial activity against a range of both gram positive and gram negative pathogenic bacterial strains. Similar result was observed with the hemolymph of some brachyuran crabs against clinical pathogens [18,19], *Thalamita crenata* [20] and *Charybdis lucifera* [21].

In the present study, maximum zone of inhibition was exhibited by the hemolymph extract of *H. araneus* against *Shigella flexineri*. But, the bacteria *Klebsiella* sp., Multi-drug resistant bacterial strain *Salmonella typhi* and fungal strains *Rhodotorula* sp., *Candida albicans*, *Aspergillus niger* were insensitive to all the hemolymph and hemocyte samples. It is interesting that the crude extracts of

hemolymph and hemocytes tested are showing very good activity against bacterial strains when compared to multi-drug resistant bacteria and fungal strains.

There are reports of good antibacterial activity by *H. araneus* (spider crab) hemocytes and plasma against *E. coli*, *Vibrio anguillarum*, *Corynebacterium glutamicum* and *S. aureus* but they are less susceptible to the extracts of gills, internal organs and exoskeleton of *H. araneus* [11]. The active fractions were resistant to heat indicative of several compounds being responsible for the antibacterial activity detected. However, several antimicrobial peptides have been isolated and partially characterized from the hemocytes of the small spider crab *H. araneus*. These include hyasin a proline rich, crustins [22] and arasin-1 a proline-arginine rich [23], crustin isoforms being antibacterial as well as antifungal, with highest activity against Gram-positive bacteria *C. glutamicum*. Though antimicrobial activity against few selected microorganism is reported for the plasma of *H. araneus*, there are no reports of any investigation on the type of compounds responsible for the activity.

In the present investigation, methanolic extract of *H. araneus* hemolymph and hemocytes samples were evaluated against 16 different, pathogenic bacterial, fungal and multi drug resistant bacterial strains and the positive active hemolymph extract was further subjected to NMR and mass spectrometry studies. It is evident from spectral data that antimicrobial activity observed in the extract is due to the lipids present. The hemolymph extract showed the presence of fatty acids, fatty esters as the major components and glycerides and glycolipids as the minor constituents of the hemolymph.

The antibacterial activity of lipids particularly the activity of fatty acids and monoglycerides have been extensively studied by Kabara and co-workers [24-26]. They demonstrated that of all the saturated fatty acids studied ranging in chain from C₆-C₁₈, lauric acid (C₁₂) was the most potent against Gram-positive bacteria tested, that that addition of a double bond to long chain saturated fatty acids enhanced antibacterial activity with mono-enoic acid,oleic acid (C_{18:1}) being more inhibitory than the corresponding saturated fatty acid (stearic acid), but less active than dienoic derivatives (C_{18:2}), with palmitoleic acid and linoleic acid exhibiting maximum against Gram-positive bacteria, that monoglycerides of medium chain saturated FAs, particularly monolaurin, are more active than the FFAs and that FFAs have low activity against Gram-ve bacteria . This has in fact been confirmed by similar studies by Bergson and his group [27] who found that lauric, palmitoleic and monocarpin reduces streptococci colony forming units one million fold or grater in 10 minutes, whereas other FFAs (free

fatty acids) and monoglycerides had small or negligible effects. Same group [28] also studied the susceptibility of *Candida albicans* to several fatty acids and their 1-glycerides. They observed that capric acid, 10 carbon saturated fatty acid, causes the fastest and most effective killing of all the three strains of *C. albicans* tested. Lauric acid, a 12 carbon saturated fatty acid, was the most active acid at lower concentrations. Subsequently, Frentzen *et al.*, [29] reported on the medium chain fatty acids of 8-12 carbon atoms exhibiting antibacterial and antifungal properties, which are enhanced when these acids are esterified with glycerol [29].

It has generally been observed that Gram-positive bacteria are more susceptible to the antibacterial effect of lipids than Gram-negative bacteria. However, there are many exceptions to this generalization. Besides there are reports [30,31] of Gram-negative bacteria *Escherichia coli* and *Salmonella spp.* being extremely resistant to antibacterial lipids at neutral pH, but are killed at acid pH. *E.coli* is also susceptible to the combined effect of lipids at high temperature [30] or by monocarpin or monolaurin in the presence of citric acid. Acid or high temperature seems to act by removing a permeability barrier in the outer membrane of these bacteria.

In the present investigation, as mentioned above, spectral data of the methanolic extract of hemolymph, was indicative of the presence lipids particularly, fatty acids, fatty esters as the major components and glycerides and glycolipids as the minor components of the hemolymph.

Therefore it is quite likely that the activity observed in the present work is partly due to the presence of fatty acids, oleic and linoleic acids. Further, the activity reported [11] for *H. araneus* is against few selected species which includes *E. coli* and *S. aureus*. In the present investigation, hemocyte samples of all the crabs except *Charybdis helleri* were effective against *S. aureus* whereas only the hemolymph of *H. araneus*, *Podophthalmus vigil* and *Portunus sanguinolentus* showed activity against *E.coli*, the hemolymph of remaining crabs being ineffective against it. Our results are well in agreement with the observations of Haug and co-workers [11] for *H.araneus*. There are no reports in the literature on the antimicrobial activity in the hemolymph/hemocytetes of the remaining five brachyuran crabs of the present study. It is being reported here for the first time. Regarding the antimicrobial properties of glycolipids there is a solitary reference in the literature on the mild antibacterial and antifungal properties of glycolipids identified from red alga *Chondria armata* [32]. Detailed studies on antimicrobial properties and spectral characterization of active principle of *H. araneus* is the future

objective of this work.

It is encouraging to know that a few lipids have shown potential and desirable therapeutic properties like antibacterial and antifungal activities [33] Also they have been shown to protect against topical and systemic infections in combination with conventional antibiotics

Recently marine derived antimicrobial compounds have opened a new perspective for pharmaceutical developments. The present study clearly shows that the hemolymph of brachyuran crab exhibit significant antimicrobial activity and more detailed studies might lead to novel structures with promising potential as antimicrobial agents.

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Table 1 Antimicrobial activity of methanolic extracts of crab hemolymph and hemocytes against various pathogenic strains.

Sample		B1	B2	B3	B4	B5	B6	B7	D1
10µl/disk		<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>	<i>Salmonella typhi</i>	<i>Shigella flexineri</i>	<i>Klebsiella sp.</i>	<i>Vibrio cholerae</i>	<i>Streptococcus pyogenes</i>
<i>Hyas araneus</i>	Crabs	+	1	+	1	6	-	4	1
<i>Podophthalmus vigil</i>	hemolymph	+	+	+	+	-	-	3	1
<i>Dromia dehanni</i>		-	+	+	+	-	-	2	+
<i>Charybdis helleri</i>		-	+	-	+	-	-	1	+
<i>Portunus sanguinolentus</i>		+	-	+	+	-	-	3	+
<i>P. pelagicus</i>		-	-	+	+	-	-	-	-
<i>Hyas araneus</i>	Crab hemo	-	-	+	1	-	-	4	-
<i>Podophthalmus vigil</i>	cytes	-	-	+	+	-	-	2	-
<i>Dromia dehanni</i>		-	+	+	3	1	-	4	-
<i>Charybdis helleri</i>		-	+	+	+	-	-	2	-
<i>Portunus sanguinolentus</i>		-	-	+	+	+	-	1	-
<i>P. Pelagicus</i>		-	-	+	+	-	-	1	-
Penicilin G 10 µg/ disk		5	1	1	3	5	-	6	5
Ketoconazole 10 µg/ disk									

Numbers in the table indicate the zone of inhibition in millimeters from the center of imbued disk
 - indicates no activity, + indicates weak activity

Table 1 (continued).

Sample 10µl/disk	D2 <i>Acinetobacter</i> sp	D3 <i>Salmonella</i> <i>typhi</i>	D4 Methicillin-resistant <i>Staphylococcus aureus</i>	F1 <i>Aspergillus</i> <i>fumigatus</i>	F2 <i>Rhodotorula</i> sp.	F3 <i>Candida</i> <i>albicans</i>	F4 <i>Cryptococcus</i> <i>neoformans</i>	F5 <i>Aspergillus</i> <i>niger</i>
<i>Hyas araneus</i>	1		-	-	-	-	+	-
<i>Podophthalmus vigil</i>	+	-	-	+	-	-	+	-
<i>Dromia dehanni</i>	+	-	-	+	-	-	-	-
<i>Charybdis helleri</i>	-	-	-	+	-	-	-	-
<i>Portunus</i> <i>sanguinolentus</i>	+	-	-	-	-	-	-	-
<i>P. pelagicus</i>	+	-	-	-	-	-	-	-
<i>Hyas araneus</i>	+	-	-	+	-	-	-	-
<i>Podophthalmus vigil</i>	-	-	-	+	-	-	-	-
<i>Dromia dehanni</i>	+	-	-	+	-	-	-	-
<i>Charybdis helleri</i>	+	-	-	-	-	-	-	-
<i>Portunus</i> <i>sanguinolentus</i>	+	-	+	+	-	-	-	-
<i>P. Pelagicus</i>	+	-	+	-	-	-	-	-
Penicilin G 10 µg/ disk	4	-	3					
Ketoconazole 10 µg/disk				1-2	-	1	1	-

Figure captions

Figure 1. Infrared spectrum of the methanolic extract of the crab *H. araneus*

Figure 2. ^1H NMR spectrum (CDCl_3) of the methanolic extract of hemolymph of *H. araneus* Figure 3. ^{13}C NMR spectrum (CD_3OD) of the methanolic extract of hemolymph of *H. araneus* Figure 4. Positive ESI-MS of the infused extract of hemolymph of *H. araneus* Figure 5. Positive ESI-MS/MS of the molecular species with $[\text{M}+\text{H}]^+$ at m/z 461 Figure 6. Positive ESI-MS/MS of the molecular species with $[\text{M}+\text{H}]^+$ at m/z 475

Figure 7. Structure of the glycolipid with molecular mass of 475 amu.