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Antimicrobial Lipids from the Hemolymph of Brachyuran Crabs

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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, the highest zone of inhibition was exhibited by both hemolymph and hemocytes samples against *Vibrio cholerae*. On the basis of TLC, ^{1}HNMR, and ^{13}CNMR 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 *H. 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 and 246, due to fatty acids/esters present in the sample.

Keywords Crab · Hemolymph · Lipid · Antimicrobial · TLC · ESI-MS

Abbreviations

TLC	Thin layer chromatography
^{1}H NMR	Proton nuclear magnetic resonance
^{13}CNMR	Carbon 13 nuclear magnetic resonance
m/*z*	Mass-to-charge
ESI-MS	Electrospray ionization mass spectrometry
mm	Millimeter
cm	Centimeter
MS/MS	Tandem mass spectrometry

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NaCl	Sodium chloride
pH	Hydrogen ion concentration
w/v	Weight/volume
V/V	Volume of the substance
μl	Microliter
μg	Microgram
FTIR	Fourier-transform infrared spectroscopy
MeOH	Methanol
H_2O	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 prophylatic and therapeutic dosage is considered [1, 8].

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 the 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 2000 rpm 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 h.

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*, *S. typhi*, *S. flexineri*, *Klebsiella* sp., *Vibrio cholerae*, *Aspergillus fumigatus*, *Rhodotorula* sp., *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus niger*, *Streptococcus pyogenes*, *Acinetobacter* sp., *S. 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 μl) 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 disks (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 disks were loaded each with 10 μL of the extract by first applying 5 μL with the pipette, allowed to evaporate, then applying another 5 μL, then drying again. The disks were then placed individually using a sterile forceps in appropriate grids which were marked on the undersurface of the plated Petri plates and kept for incubation at room temperature (27°C±2) for 24 h. 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 disks: Penicillin G (Himedia), 10 μg/disk; ketoconazole (Himedia) 10 μg/disk. Four (4) disks were placed in each agar test plate (in some instances, as much as seven) by using a sterile forceps 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 millimeters using a scale.

Thin-Layer Chromatography

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 sulfuric acid as detecting agent followed by heating at 100°C till the spots were visible. Heating for a longer period turn the spots black.

General Experimental Procedures

Infra-red spectrum was recorded on Shimadzu FTIR 8201 PC spectrometers. NMR (^{1}H, ^{13}C) data were obtained on Bruker Avance 300 spectrometer with TMS as internal standard. EI-MS 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: H_2O containing trace of 0.1% TFA. The solution was directly infused at the constant flow rate of 10 μl/min into the ion-spray source using an integrated syringe pump. The declustering potential and the collision energy (25–40 V) 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*, *Podopthalmus vigil*, *Dromia dehanni*, *Charybdis helleri*, *Portunus sanguinolentus*, *Portunus 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 *H. araneus* against *S. flexineri*. Interestingly, *S. aureus* and *S. typhi* were susceptible to all the hemolymph and hemocytes samples; the latter being more sensitive to hemocytes of *D. dehanni*. *V. cholerae* seemed to be the most sensitive microorganism to crude methanolic extracts of hemolymph as well as hemocytes samples of all the six brachyuran crabs except the hemolymph of *P. pelagicus* of the present investigation.

The extracts were ineffective against the bacteria *Klebsiella* sp and multi-drug-resistant *S. typhi* and fungal strains, *Rhodotorula* sp., *C. albicans*, and *A. 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 3,100 and

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

Sample 10μl/disk		B1 *Escherichia coli*	B2 *Pseudomonas aeruginosa*	B3 *Staphylococcus aureus*	B4 *Salmonella typhi*	B5 *Shigella flexineri*	B6 *Klebsiella* sp.	B7 *Vibrio cholerae*	D1 *Streptococcus pyogenes*
Hyas araneus	Crabs hemolymph	+	1	+	1	6	−	4	1
Podopthalmus vigil		+	+	+	+	−	−	3	1
Dromia dehanni		−	+	+	+	−	−	2	+
Charybdis helleri		−	+	−	+	−	−	1	+
Portunus sanguinolentus		+	−	+	+	−	−	3	+
P. pelagicus		−	−	+	+	−	−	−	−
Hyas araneus	Crab hemo cytes	−	−	+	1	−	−	4	−
Podopthalmus vigil		−	−	+	+	−	−	2	−
Dromia dehanni		−	+	+	3	1	−	4	−
Charybdis helleri		−	+	+	+	−	−	2	−
Portunus sanguinolentus		−	−	+	+	+	−	1	−
P. Pelagicus		−	−	+	+	−	−	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 *Acinetobacter* sp	D3 *Salmonella typhi*	D4 Methicillin-resistant *Staphylococcus aureus*	F1 *Aspergillus fumigatus*	F2 *Rhodotorula* sp.	F3 *Candida albicans*	F4 *Cryptococcus neoformans*	F5 *Aspergillus niger*
Hyas araneus	1	−	−	−	−	−	+	−
Podopthalmus vigil	+	−	−	+	−	−	+	−
Dromia dehanni	+	−	−	+	−	−	−	−
Charybdis helleri	−	−	−	+	−	−	−	−
Portunus sanguinolentus	+	−	−	−	−	−	−	−
P. pelagicus	+	−	−	−	−	−	−	−
Hyas araneus	+	−	−	+	−	−	−	−
Podopthalmus vigil	−	−	−	+	−	−	−	−
Dromia dehanni	+	−	−	+	−	−	−	−
Charybdis helleri	+	−	−	−	−	−	−	−
Portunus sanguinolentus	+	−	+	+	−	−	−	−
P. Pelagicus	+	−	+	−	−	−	−	−
Penicilin G 10 μg/disk	4	−	3					
Ketoconazole 10 μg/disk				1–2	−	7	1	−

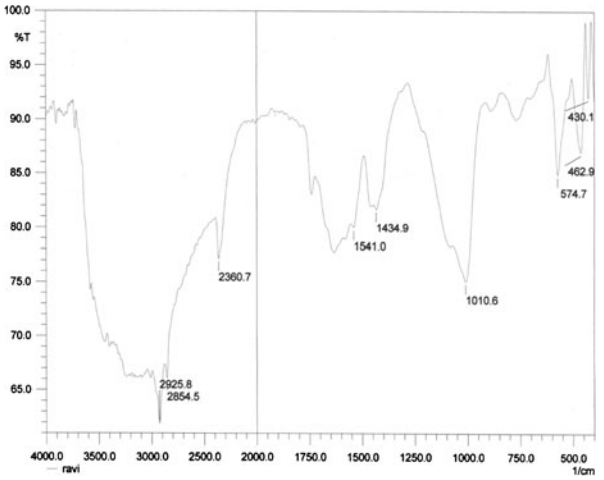


Fig. 1 Infrared spectrum of the methanolic extract of the crab *H. araneus*

3,600 cm^{-1} and 1010 cm^{-1} due to the hydroxyl functionality. A pronounced signal at 1,750 cm^{-1} was assigned to the carbonyl group of the ester. Unsaturation is evident by the presence of signals at 3,030 cm^{-1} and the broad absorption centered at ~1,650. The peaks at 2,925 and 2,854 cm^{-1} for aliphatic CH stretching were also present in the spectrum.

The ^{1}HNMR spectrum (Fig. 2) was typical of lipids with special reference to fatty acids and glycerolipids. It exhibited two terminal methyl signals, as triplet at δ 0.874 (3H, t, J= 6.9 Hz) due to the presence of saturated fatty acyl chain and a terminal methyl residue at δ 0.915 (3H, t, J=6.9 Hz) of unsaturated fatty acyl chain; a broad signal at δ 1.274 was assigned to the methylene protons of the aliphatic chains of the two acyl groups; multiplets

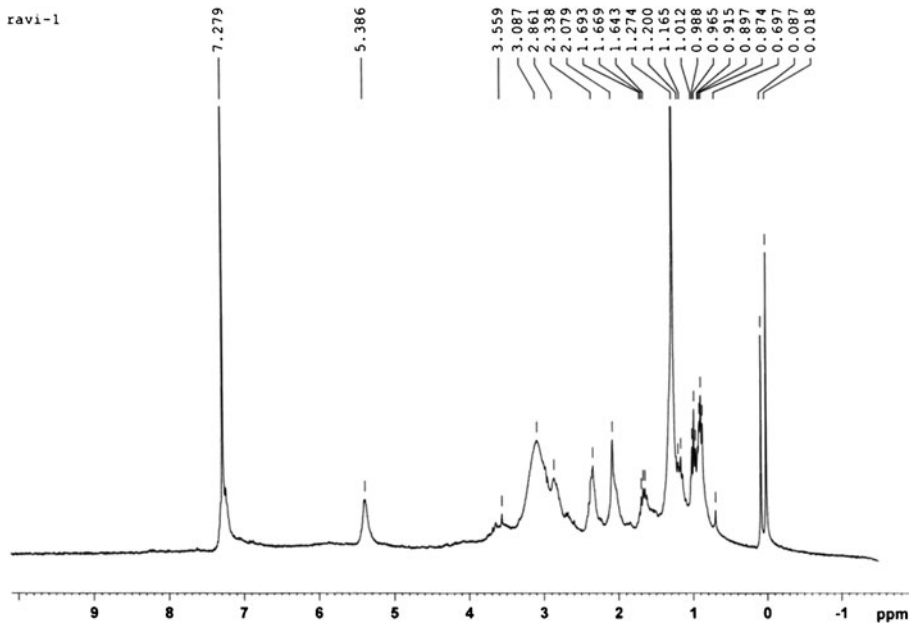


Fig. 2 ^{1}HNMR spectrum ($CDCl_3$) of the methanolic extract of hemolyph of *H. araneus*

centered at δ 2.338, 2.079 and 1.643 were attributed to the methylene groups linked α, β, and γ, respectively, to the ester/acid functional group; a triplet at δ 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 methine protons were evident at δ 5.38. The sugar protons were apparent in the region δ 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 ^{1}HNMR and though there is overlapping, this is evident in the present spectrum, with a signal centered at δ 1.00 (d, J=7.2 Hz) and the corresponding absorption in ^{13}CNMR at 17.7 ppm.

The ^{13}CNMR spectrum of the sample (Fig. 3) showed two quaternary carbons at δ 172.5 and δ 173.8, and additional two at δ 178.1 and δ 180.2; the 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 δ 14.06–20.5, methylene carbons were evident in the region between δ 20.5 and 34.22. The unsaturation of the aliphatic chain was evident between δ 125.3–129.32 with the most intense signals in this region being present at δ 127.0, δ 128.4, δ 128.9 and δ 129.3. This indicates that the glycolipids contain an acyl group wherein the fatty acids present belong to the 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 the presence of glycerides evidenced by the presence of ^{13}CNMR 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 ^{13}CNMR of the sample.

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 ESI-MS (Fig. 4) profile of the methanolic extract of hemolyph of crab *H. araneus.* ESI-MS shows cluster of peaks in the region *m*/*z* 445 to *m*/*z* 491 due to lysoglycerolipids

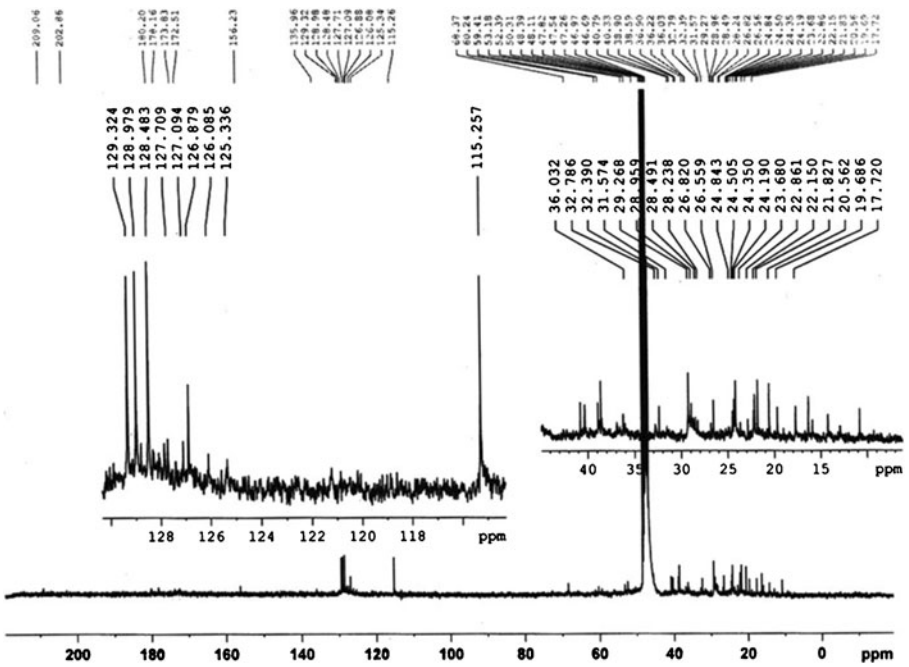


Fig. 3 ^{13}C NMR spectrum (CD_3OD) of the methanolic extract of hemolyph of *H. araneus*

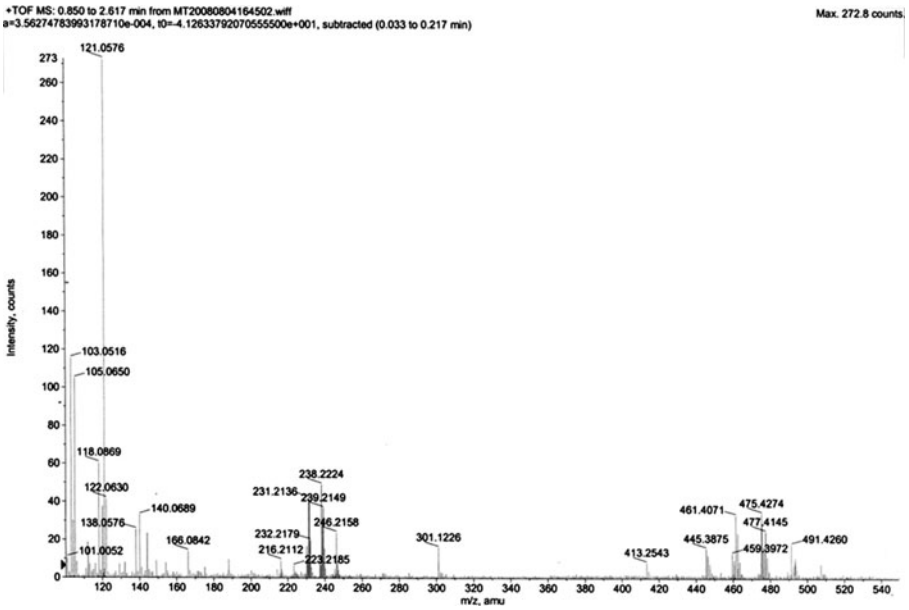


Fig. 4 Positive ESI-MS of the infused extract of hemolyph of *H. araneus*

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

Tandem mass spectra (Figs. 5 and 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* 222 in the former and at *m/z* 236 in the latter) and also due to the loss

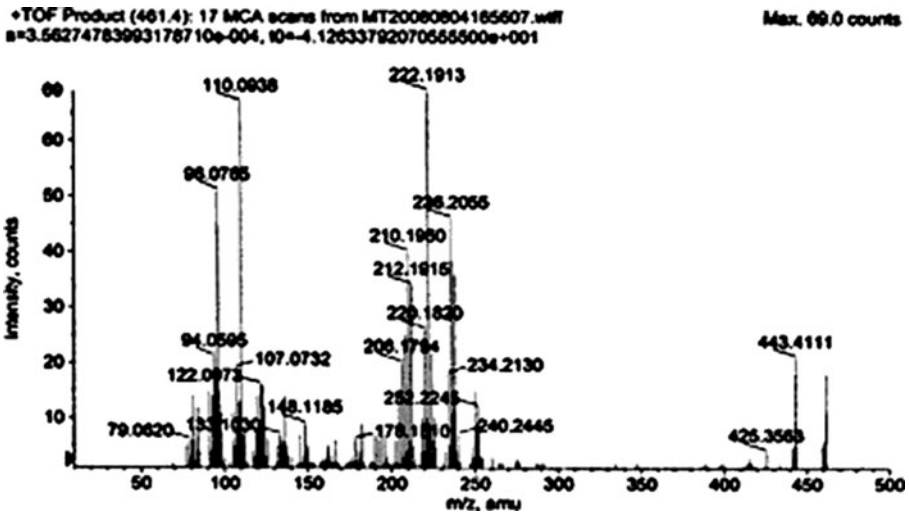


Fig. 5 Positive ESI-MS/MS of the molecular species with $[M+H]^+$ at *m/z* 461

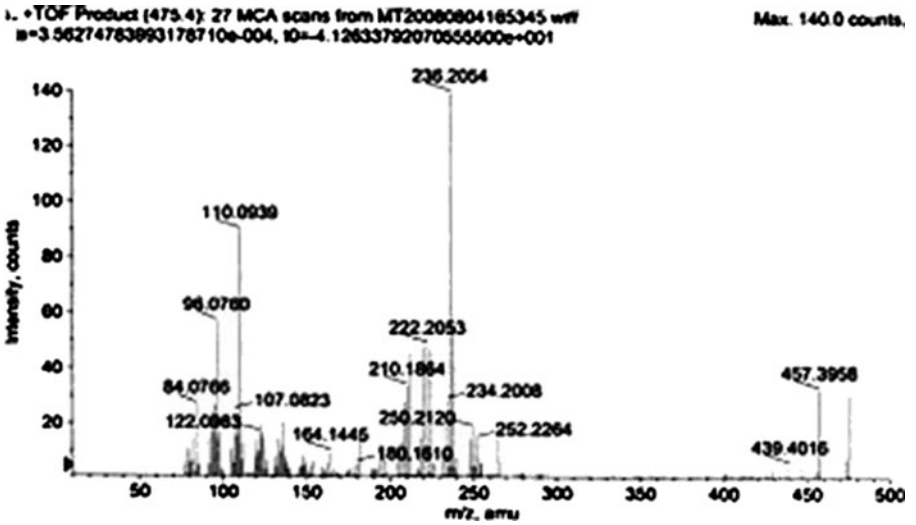


Fig. 6 Positive ESI-MS/MS of the molecular species with $[M+H]^+$ at *m*/*z* 475

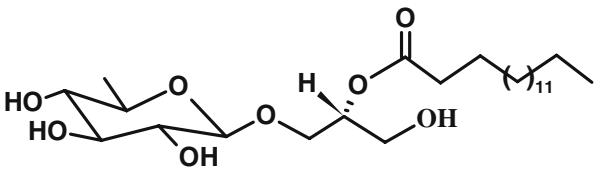
of palmitic acid. Since the cleavage at the sn-2 position is preferred based on the intensity of the signal the palmitate group was placed at position C-2 of the glycerol moiety [13, 14]. The signal at *m*/*z* 164 was attributed to protonated hexose moiety, probably rhamnosyl and not galactosyl/glucosyl 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 the above data, the glycolipid with *m*/*z* 475 has been identified as 3*O*-rhamnosyl-2-palmitoyl glycerol and assigned structure as shown in Fig. 7.

In the ESI-MS of methanolic extract of hemolyph 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 3*O*-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 microorganisms [15–17]. However, the research on marine organisms is still in its infancy considering the vast resources that still remains untapped with the majority of marine organisms yet to be screened for the discovery of useful antibiotics.

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



In the present study, the methanolic extract of crab hemolymph showed antimicrobial activity against a range of both Gram-positive and -negative pathogenic bacterial strains. A 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, the maximum zone of inhibition was exhibited by the hemolymph extract of *H. araneus* against *S. flexineri*. But the bacteria *Klebsiella* sp., multi-drug-resistant bacterial strain of *S. typhi* and fungal strains *Rhodotorula* sp., *C. albicans*, and *A. niger* were insensitive to all the hemolymph and hemocyte samples. It is interesting that the crude extracts of hemolymph and hemocytes tested show 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 anguilarum*, *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 crustin [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, has been extensively studied by Kabara and co-workers [24–26]. They demonstrated that of all the saturated fatty acids studied ranging in chain from C_6–C_{18}, lauric acid (C_{12}) was the most potent against Gram-positive bacteria tested, 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-negative bacteria. This has in fact been confirmed by similar studies by Bergsson et al. [27] who found that lauric, palmitoleic, and monocarpin reduces streptococci colony forming units one-million-fold or greater in 10 min, whereas other FFAs (free fatty acids) and monoglycerides had small or negligible effects. The same group [28] also studied the susceptibility of *C. albicans* to several fatty acids and their 1-glycerides. They observed that capric acid, a ten-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 eight to 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 and 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 a few selected species, including *E. coli* and *S. aureus*. In the present investigation, hemocyte samples of all the crabs except *C. helleri* were effective against *S. aureus*, whereas only the hemolymph of *H. araneus*, *P. vigil*, and *P. sanguinolentus* showed activity against *E. coli*, the hemolymph of remaining crabs were ineffective against it. Our results are well in agreement with the observations of Haug et al. [11] for *H. araneus*. There are no reports in the literature on the antimicrobial activity in the hemolymph/hemocytes 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* are 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 crabs exhibit significant antimicrobial activity and more detailed studies might lead to novel structures with promising potential as antimicrobial agents.

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References

1. Thormar, H., & Hilmarsson, H. (2007). *Chemistry and Physics of Lipids, 150*, 1–11.
2. Miyata, T., Tokunaga, F., Yoneya, T., Yoshikawa, K., Iwanaga, S., Niwa, M., et al. (1989). *Journal of Biochemistry, 106*, 663–668.
3. Kabara, J. J. (1978). Fatty acids and derivatives as antimicrobial agents review. In J. J. Kabara (Ed.), *Symposium on the pharmacological effect of lipids* (pp. 1–14). Champaign, IL, U.S.A: The American Oil Chemists Society.
4. Sands, J. A., Auperin, D. D., Landin, P. D., Reinhardt, A., & Cadden, S. P. (1978). Antiviral effects of fatty acids and derivatives: lipid-containing bacteriophages as a model system. In J. J. Kabara (Ed.), *Symposium on the pharmacological effect of lipid* (pp. 75–95). The American Oil Chemists_Society: Champaign, IL, USA.
5. Snipes, W., & Keith, A. (1978). Hydrophobic alcohols and di-*tert*-butyl phenols as antiviral agents. In J. J. Kabara (Ed.), *Symposium on the pharmacological effect of lipid* (pp. 63–73). Champaign, IL, U.S.A: The American Oil Chemists I Society.
6. Anon (2002) Undecylenic Acid. *Monograph Altern Med Rev*, *7*, 68–70.

7. Kongtawelert, P. (1998). *Molecular marine biology and biotechnology, 7*, 280–286.
8. Haug, A., Høstmark, A. T., & Harstad, O. M. (2007). *Lipids Health Dis, 6*, 25.
9. Chisholm, J. R. S., & Smith, V. J. (1995). *Comparative Biochemistry and Physiology, 110*, 39–45.
10. Schnapp, D., Kemp, G. D., & Smith, V. J. (1996). *European Journal of Biochemistry, 240*, 532–539.
11. Haug, T., Kjuul, A., Stensvag, K., Sandsdalen, E., & Styrvold, O. (2002). *Fish & shellfish immunology, 12*, 371–385.
12. Bauer, A. W., Kirby, W. M. M., Sherris, J. C., & Turck, M. (1996). *American journal of clinical pathology, 45*, 493–496.
13. Blair, I. A. (1990). *Methods in Enzymology, 187*, 13–23.
14. Waugh, R. J., & Murphy, R. C. (1996). *Journal of the American Society for Mass Spectrometry, 7*, 490–499.
15. Rinehart, K. L., Shaw, P. D., Shield, L. S., Gloer, J. B., Harbour, G. C., Koker, M. E. S., et al. (1981). *Pure and applied chemistry, 53*, 795–817.
16. Ravichandran, S., & Rameshkumar, G. (2006). *Seshaiyana, 14*, 12–15.
17. Ravichandran, S., Kathiresan, K., & Balaram, H. (2007). *Biotech Mole Biol Reviews, 2*, 33–38.
18. Veeruraj, A., Ravichandran, S., & Rameshkumar, G. (2008). *Trends in Appl Sci Res, 3*, 174–181.
19. Veeruraj, A., Ravichandran, S., & Rameshkumar, G. (2008b). *Aqua Biol Aqua*, pp. 46–57.
20. Rameshkumar, G., Ravichandran, S., Kaliyavarathan, G., & Ajithkumar, T. T. (2009). *World J Fish & Marine Sci, 1*, 74–79.
21. Rameshkumar, G., Ravichandran, S., & Aravindhan, T. (2009). *Middle East J Sci Res, 4*, 40–43.
22. Sperstad, S. V., Haug, T., Paulsen, V., Rode, T. M., Strandskog, G., Solem, S. T., et al. (2009). *Developmental and Comparative Immunology, 33*, 583–591.
23. Stensvag, K., Sperstad, S. V., Rekdal, O., Indrevoll, B., & Styrvold, O. B. (2008). *Developmental and Comparative Immunology, 32*, 275–285.
24. Kabara, J. J., Swieczkowski, D. M., Conley, A. J., & Truant, J. P. (1972). *Antimicrobial Agents and Chemotherapy, 2*, 23–28.
25. Conley, A. J., & Kabara, J. J. (1972). *Antimicrobial Agents and Chemotherapy, 4*, 501–506.
26. Kabara, J. J. (1975). *Cosm Perfum, 90*, 21–25.
27. Bergsson, G., Arnfinnsson, J., Steingrimsson, O., & Thormar, H. (2001). *APMIS, 109*, 670–678.
28. Bergsson, G., Arnfinnsson, J., Steingrimsson, O., & Thormar, H. (2001). *Antimicrobial Agents and Chemotherapy, 45*, 3209–3212.
29. Frentzen, M., Weier, D., & Feussner, I. (2003). *European Journal of Lipid Science and Technology, 105*, 784–792.
30. Bergsson, G., Steingrimsson, O., & Thormar, H. (2002). *J Antimicrob Agents, 20*, 258–262.
31. Thormar, H., Hilmarsson, H., & Bergsson, G. (2006). *Applied and Environmental Microbiology, 72*, 522–526.
32. Al-Fadhli, Ammar, Wahidulla, Solimabi, & D'Souza, Lisette. (2006). *Glycobiology, 16*, 902–915.
33. Fielding, R. M., & Lasic, D. D. (1999). *Expert Opinion in Therapeutic Patent, 9*, 1679–1688.