

## Antimicrobial Activity from the Hemolymph of the Hermit Crab *Clibanarius clibanarius* (Herbst 1791)

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**Abstract:** Marine organisms represent an excellent source for bioactive compounds. The field of marine natural products has been expanding in response to the growing number of structurally novel and bio medically promising natural products being isolated from the marine source. In the present study antimicrobial activity of the hemolymph of hermit crabs *Clibanarius clibanarius* was investigated. In antibacterial activity the highest zone of inhibition was observed in the hemolymph of *C. clibanarius* against *Salmonella typhi* (16 mm) the minimum activity was observed against *Streptococcus pyogenes* (7mm). In TLC the incidence of pink spots is indicating the presence of amino acids and peptides. The amount of protein present in the hemolymph was estimated as 1.08 mg/ml. In SDS-PAGE the presences of bands were detected in the gel represents the presence of proteins in the range nearly 63 kDa. The dFT-IR spectrum reveals that the hemolymph of the crab *C. clibanarius* comprises to have peptide as their predominant chemical groups. The present study clearly shows that the various fractions were found to be rich in ninhydrin positive spots indicating the possibility of containing peptides. Hence, the present study indicated that the hemolymph of *C. clibanarius* crabs may potential antibiotics.

**Key words:** Hermit crabs • Hemolymph • Anti-microbial peptide • Thin-layer chromatography • FT-IR

### INTRODUCTION

Marine invertebrates rely solely on innate immune mechanisms that include both humoral and cellular responses. Clinical uses of antimicrobial compound were suggested many years ago by several authors [1, 2]. However, not much pharmaceutical products as active compounds have as yet been approved for clinical use as prophylactic or therapeutic drugs against bacterial infections. Some of the brachyuran crabs have shown pronounced activities, useful in the biomedical area [3, 4]. 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 [5]. Marine organisms are rich sources of structurally diverse bioactive compounds with various biological activities [6].

They offers enormous resource for novel compounds and serves as a source of functional materials [7-10] in particular, some of these bioactive compounds are proteinaceous in natural and include proteins, peptides and amino acids. Furthermore, being rich sources of protein, some marine organisms, are ideal starting

materials for the generation of protein defined bioactive peptides. Hence, a broad, based screening of marine crabs for bioactive compounds is necessary. The potential of hermit crabs as a source of biologically active products is largely unexplored. Thus it is obvious that no antimicrobial peptide study on the hermit crabs *Clibanarius clibanarius* has been attempted, hence the present study focused on the antibacterial and antifungal properties of the above mentioned crab followed by TLC Protein estimation SDS and FTIR.

### MATERIALS AND METHODS

**Sample Collection and Preparation:** Hemolymph was collected by cutting each walking legs of the animal with a fine sterile scissor. To avoid hemocyte degranulation and coagulation, the hemolymph 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 hemolymph it 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 crab hemolymph was determined against 10 different bacterial strains viz., *Staphylococcus aureus*, *Salmonella typhi*, *Salmonella paratyphi*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Escherichia coli*, *Vibrio cholera*, *Vibrio parahaemolyticus* and 10 fungal strains viz., *Aspergillus niger*, *Aspergillus flavus*, *Alternaria alternaria*, *Candida albicans*, *Epidermophyton floccosum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Penicillium* sp. *Rhizopus* sp. *Mucor* sp.

These clinical strains were obtained from the Department of Medical Microbiology, (Raja Muthiah Medical College Hospital) Annamalai University, Annamalai Nagar. The spectrum of antibacterial and antifungal activity was studied using the techniques. Antibacterial and antifungal activity was expressed in terms of zone of inhibition in mm using a scale and recorded.

**Thin Layer Chromatography:** Since in the present investigation, peptides were compounds of interest, ninhydrin was used as a detecting agent. This reagent is specific for amines. TLC plate was first developed in a butanol: acetic acid: water (B:A:W) solvent system, dried at room temperature, sprayed with ninhydrin and heated at 100°C in oven for few minutes till the colored spots were visible. Pink spots were checked for the presence of proteins.

**Protein Estimation:** The amount of protein was measured by spectrometry according to the Lowery *et al.* [11] method with different concentrations (0.1 – 0.5 mg ml<sup>-1</sup>) of Bovine Serum Albumin (BSA) as a standard. Biuret reagent as a color reactant and concentration was calculated in response to absorbance at 540nm in spectrophotometer.

**Determination of Molecular Weight by Using SDS-PAGE:** SDS-PAGE is used to find out the molecular weight active fractions of the sample. SDS-PAGE was performed in 12% separating gels, according to the method described by Laemmli [12].

**Fourier Transform-Infrared Spectroscopy (FT-IR):** FT-IR was used to identify the chemical identities in a wide range of compounds. Infrared spectroscopy was a useful analytical technique for detection of functional groups in organic compounds. Sample of 10 mg was mixed with 100 mg of dried Potassium bromide (KBr) was compressed to prepare as a salt disc (10 mm dia) for spectrometer reading further. The infrared absorption bands identified molecular components and structures.

## RESULTS

**Antibacterial Activity:** The antibacterial activity of the hemolymph of the hermit crab *C. clibanarius* was used for the present study. In antibacterial activity the highest zone of inhibition was observed in the hemolymph of *C. clibanarius* against *S. typhi* (16 mm) the minimum activity was observed against *S. pyogenes* (7mm). The positive control highest Tetracyclin showed activity against *K. oxytoca* (22.9 mm) the minimum activity observed against *E. coli* (11.8mm) (Fig. 1). The antifungal activity of the hemolymph of *C. clibanarius* against the fungal strains and there was no activity observed.

**Thin-Layer Chromatography (TLC):** Thin-Layer chromatography profiling was done for the hemolymph samples of *C. clibanarius* in solvent system was a combination of butanol, acetic acid and water (B: A; W) in proportions of 5:1:4. The plates when developed in the solvent systems showed light pink spots when sprayed in ninhydrin. The plate with fractions developed in BAW

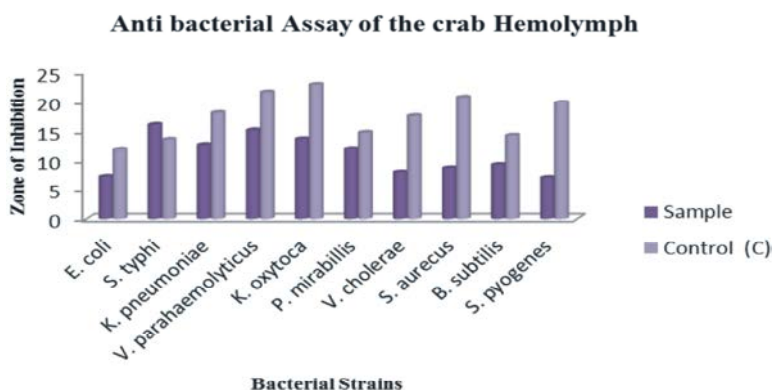


Fig. 1: Antibacterial Activity of *C. clibanarius*



Fig. 2: Thin-Layer Chromatography of *C. clibanarius*

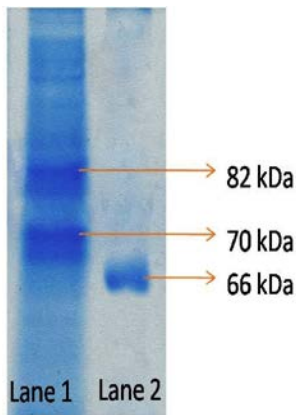


Fig. 3: SDS-PAGE of the Hemolymph

as the solvent and sprayed system and sprayed with ninhydrin, showing pink spots indicating the presence of amino acids and peptides is shown in (Fig. 2).

**Hemolymph Protein Estimation:** The protein content of the hemolymph of *C. clibanarius* was estimated using lowery method. Protein concentration of the hemolymph is measured using a spectrophotometer. The amount of protein present in the hemolymph was estimated as 1.08 mg/ml.

**Determination of Molecular Weight by Using SDS-PAGE:** The hemolymph showed antibacterial activity was subjected to SDS-PAGE to estimate the molecular weight of proteins present in it. Different standard were used to determine the molecular weight of hemolymph proteins. The stained gel revealed that the hemolymph contained a simple population of proteins. There are different molecular weight markers were used. Four bands were detected in the gel that represents the presence of proteins in the range nearly 25.7-75 k Da. The presence of bands were detected in the gel represents the presence of proteins in the range nearly 63 kDa (Fig. 3).

**Fourier Transform-Infrared Spectroscopy (FT-IR):** As a preliminary analytical screening of sponges exhibited very good antimicrobial potency were subjected for FT-IR spectral studies for screening of basic functional compounds present in it. The results of FT-IR spectrum reveal that the hemolymph of the crab *C. clibanarius* comprises to have peptide as their predominant chemical groups which are shown on (Fig. 4).

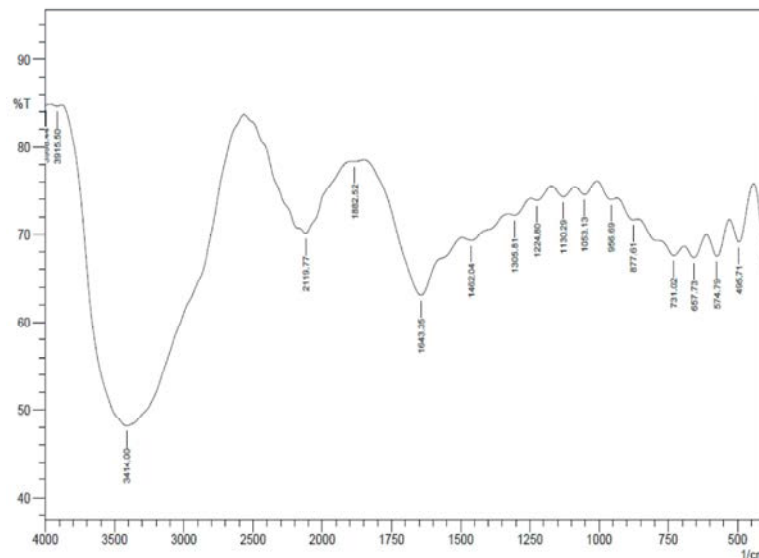


Fig. 4: FT-IR of the hemolymph

## DISCUSSION

In recent years, great attention has been paid to study the bioactivity of natural products due to their potential pharmacological utilization. The present research investigation is made on the basis of in search of antimicrobial peptides from the serum of *C. clibanarius* hermit crab collected from the Parangipettai coastal environment. In the present study, hemolymph of the hermit crab *C. clibanarius* showed antibacterial activity against a range of both Gram-positive and -negative pathogenic bacterial strains. Haug *et al.* [5] found the antibacterial activity in different body-parts of *Pagurus bernhardus* (Hermit crab), *Pandalus borealis* (Northern shrimp), *Hyas araneus* (Spider crab) and *Paralithodes camtschatica* (King crab). A similar result was observed with the hemolymph of some brachyuran crabs against clinical pathogens [13-19].

In the current examination hemolymph of *C. clibanarius* showed maximum antibacterial activity against *V. parahaemolyticus* (15.1mm). Li *et al.* [20] and Ma *et al.* [21] induced *Bullacta exarata* with *E. coli*, isolated peptides from its mucus and lymph and found marked antimicrobial activity against *E. coli* and *S. aureus*. All these findings confirm the idea that endogenous antimicrobial peptides in marine invertebrates could be good resources for new antimicrobial compounds. The hemocytes of the shore crab, *Carinus maenas*, has been shown to contain potent antibacterial activity against a variety of gram positive and gram negative bacteria [22].

In the present investigation the percentage of protein concentration of hemolymph the crab *C. clibanarius* was recorded as 2.61 mg/ml. Similar type of study was carried out in various brachyuran crabs viz., *S. serrata* and *S. tranquebarica* followed by *Nanoepisesarma minutum*, *Neoepisesarma tetragonum*, *Metapograpsus maculatus*, *Macrophthalmus depressus*, *T. crenata*, *Charybdis ferriata*, *C. lucifera*, *C. aboensis*, *C. natator*, *Portunus pelagicus*, *T. crenata* and *Grapsus strigosus* [18,23].

The concentration of protein in the hemolymph shows wide inter specific variation among decapod crustaceans, ranging from as low as 28mg/ml in *C. maenas* [24] to as high as 222mg/ml in *Uca minax* [25] of this hemolymph protein, consists of the respiratory pigment haemocyanin [26, 27]. Rameshkumar *et al.* [18] reported female crab *Charybdis lucifera* hemolymph and 25KDa proteins of male hemolymph crab of *C. lucifera*. Two clear bands were detected in the gel which represents proteins of molecular weight 45KDa and 25KDa.

In the current investigation four bands were detected in the gel that represents the presence of proteins in the range nearly 25.7-75 k Da. The presence of bands were detected in the gel represents the presence of proteins in the range nearly 63 kDa. Likewise Mercy and Ravindranath, 1993 purified agglutinin and characterized protein from the hemolymph of the marine crab, *Scylla serrata* by SDS polyacrylamide gel electrophoresis. Also Jayasankar and Subramanian [29] operated that a 20KDa a protein from the seminal fluid of *S. Serrata* was found to have anti microbial activity against commonly occurring marine bacteria. Recently marine peptides have opened a new perspective for pharmacological developments. The present study clearly indicates that the hemolymph of *C. clibanarius* crabs would be a good source of bioactive substance and would replace the existing in adequate and cost effective antibiotics and clearly indicates antimicrobial peptides isolated from *C. clibanarius* would be a good source of bioactive substance and would replace the existing in adequate and cost effective antibiotics.

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