ANTIMICROBIAL AND ANTIOXIDANT PROTEINS FROM THE CRAB LIAGORE RUBROMACULATA (DE HAAN, 1835)

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ABSTRACT

The emergence of new infectious diseases and resistance to the antibiotics by the existing ones led to the new sources for drug discovery. In the present study antibacterial and antioxidant activity of the hemolymph of brachyuran crabs Liagore rubromaculata was investigated. In the antibacterial activity against 6 pathogenic strains maximum zone of inhibition was recorded against highest zone of inhibition was observed in the hemolymph of female crab L. rubromaculata against P. vulgaris(12 ± 1 mm) and the minimum activity was observed against E.coli(6 ± 1 mm). In the hemolymph of male crab L. rubromaculata the highest zone of inhibition was observed Against E. faecalis(9 ± 1 mm) and the minimum activity observed against K. oxytoca (7mm). In TLC appearance of pink spots indicates the presence of protein in the tested sample. In SDS PAGE, in male four bands were detected in the gel that represents the presence of proteins in the range nearly 26.5-73 kDa. In female five bands were detected in the gel that represents the presence of proteins in the range nearly 25.2-75 k Da. Hence the present study indicated that the hemolymph of L. rubromaculata crabs may potential antibiotics.

KEYWORDS: Antimicrobial proteins, DPPH, Hemolymph, TLC, SDS.

INTRODUCTION

Antimicrobial peptide defense in crustacean has long been suspected. In 1972, bactericidal activities were observed in Lobster Homarus americanus plasma[1] and hepatopancreas[2]. However, the high diversity of the variants found in most crustacean AMP classes can presumably confer a broad spectrum of activity to a single AMP family. Homarin is a short linear peptide isolated from the hemocytes of the American lobster Homarus americanus and does not contain any specific abundant amino acid residues [3]. Curiously, in crustaceans,
defensin members were only very recently identified in the Japanese spiny lobster *Panulirus japonicas* using an expressed sequence tag (EST) approach [4]. Humoral immunity in marine invertebrates is characterized by antimicrobial agents present in the blood cells and plasma along with reactions such as hemolymph coagulation or melanization [5,6]. The multimeric coagulation and phenoloxidase systems are also considered to be important defenses in these organisms. Other factors described as part of the immune system include agglutinins, hemolysins, lysozyme and antimicrobial factors [7, 8].

The purification and characterization of a proline-rich antibacterial peptide, with sequence similarity to bactenecin-7, from the hemocytes of the shore crab, *Carcinus maenas* were studied by [9]. Partially characterized a cystine-rich 11.5kDa gram-positivespecific antimicrobial peptide from *C. maenas* [10]. Callinectin is a cationic antimicrobial peptide of 3.7 kDa that represents the major antibiotic activity from the blue crab *Callinectes sapidus* [11]. Some of the brachyuran crabs have shown pronounced activities and may be useful in the biomedical area. The potential of marine crabs as source of biologically active products is largely unexplored. Hence, a broad based screening of marine crabs for bioactive compounds is necessary. Thus it is obvious that no antimicrobial peptide study on the *L. rubromaculata* crabs has been attempted, hence the present study focused on the antibacterial and antioxidant properties of the above mentioned crab followed by TLC Protein estimation and SDS.

**MATERIALS AND METHODS**

**Collection of Hemolymph**

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’s hemolymph was determined against 6 bacterial strains viz., *Vibrio cholera*, *Salmonella typhi*, *Escherichia coli*, *Klebsiella oxytoca*, *Proteus vulgaris*, And *Enterobacter faecalis*. These pathogens strains were obtained from the Department of medical microbiology (Raja muthiyah medical college and hospital) Annamalai University, Annamalainagar.(Table.1)
Table.1 Characteristics of Microbial strains tested

<table>
<thead>
<tr>
<th>S.No</th>
<th>Microbial Strains tested</th>
<th>Characteristics of Microbial strains tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Vibrio cholera,</em></td>
<td>It is a &quot;comma&quot; shaped Gram-negative bacteria with a single, polar flagellum for movement.</td>
</tr>
<tr>
<td>2</td>
<td><em>Salmonella typhi,</em></td>
<td>It is a rod-shaped gram-negative bacterium between 2 and 5 micrometers long and 0.7 to 1.5 micrometers in diameter.</td>
</tr>
<tr>
<td>3</td>
<td><em>Escherichia coli,</em></td>
<td><em>E. coli</em> is an aerobic, gram-negative, rod shaped bacteria that cannot sporulate.</td>
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<tr>
<td>4</td>
<td><em>Klebsiella oxytoca,</em></td>
<td>It is a gram-negative bacterium with a cylindrical rod shape measuring 2 µm by 5µm</td>
</tr>
<tr>
<td>5</td>
<td><em>Proteus vulgaris,</em></td>
<td><em>Proteus vulgaris</em> is a rod-shaped, gram-negative bacterium known to cause urinary tract infections and wound infections.</td>
</tr>
<tr>
<td>6</td>
<td><em>Enterobacter faecalis,</em></td>
<td>is a Gram-positive, commensal bacterium, nonmotile, facultatively anaerobic microbe.</td>
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**Anti-Microbial Assay**

The spectrum of antibacterial was studied against six bacterial with a positive control tetracycllin. Antibacterial activity was expressed in terms of diameter of Zone of inhibition was measured in mm using Vernier caliper or a scale and recorded. Results were expressed as mean ± standard deviation

**Antioxidant Assay**

To measure the antioxidant activity of the hemolymph of crab *L.rubromaculata*, 0.1ml of the sample was taken in a vial and then adds 0.5ml of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). Then these sample mixture was incubated for 30min in a dark place. After the incubation period the DPPH scavenging activity was measured by using the spectrophotometer in 620nm.

**Thin Layer Chromatography**

TLC plate is first developed in a suitable solvent system, dried at room temperature, sprayed with ninhydrin and heated at 100°C in oven for few min till the colored spots are visible. Pink spots were checked for the presence of amino acid and amines.

**Estimation of Hemolymph Protein**

The amount of protein was measured by spectrometry according to the [12] method with different concentrations of Bovine Serum Albumin (BSA) as a standard. Biuret reagent as a color reactant and concentration was calculated in response to the absorbance at 540nm in spectrophotometer.
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 [13].

RESULTS
Antibacterial Assay
Antibacterial activity of the male and female hemolymph sample of *L. rubromaculata* was used for the present study. The zone of inhibition in different bacterial strains against *L. rubromaculata* hemolymph is shown in (Fig 1). In antibacterial activity the highest zone of inhibition was observed in the hemolymph of female crab *L. rubromaculata* against *P. vulgaris* (12 ± 1mm) and the minimum activity was observed against *E. coli* (6 ± 1mm). In the hemolymph of male crab *L. rubromaculata* the highest zone of inhibition was observed against *E. faecalis* (9 ± 1 mm) and the minimum activity observed against *K. oxytoca* (7 ± 1mm). The antibacterial agent of tetracycline showed activity against all the bacterial strains tested. The highest zone of inhibition was observed against *K. oxytoca* (22.9 ± 0.3 mm) the minimum activity observed against *E. faecalis* (11.8 ± 0.4 mm).

![Antibacterial Activity of the Crab Hemolymph](image)

**Fig 1. Antibacterial Activity of the Crab Hemolymph**

Anti-Oxidant Assay
The free radical scavenging activity of protein from crab *L. rubromaculata* hemolymph was assessed by the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. The results shows that a maximum percentage of DPPH scavenging activity was recorded 1.381% in male and the minimum was recorded as 1.003% in female crab sample.

Thin-Layer Chromatography (TLC)
Thin-Layer chromatography profiling was done for the hemolymph samples of the crab *L. rubromaculata* in solvent system which 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 showing pink spots indicating the presence of amino acids and peptides.

**Hemolymph Protein Estimation**
The protein content of the hemolymph of *L. ruberomaculata* was estimated. Protein concentration of the hemolymph is measured using a spectrophotometer. The amount of protein present in the hemolymph was estimated to as 1.62 mg/ml in male and 1.5 mg/ml in female crabs.

**Determination of Molecular Weight by Using SDS-PAGE**
The hemolymph of *L. ruberomaculata* showed antibacterial activity was subjected to SDS-PAGE to estimate the molecular weight of active 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 is different molecular weight markers were used. In male four bands were detected in the gel that represents the presence of proteins in the range nearly 26.5-73 kDa. In female five bands were detected in the gel that represents the presence of proteins in the range nearly 25.2-75 kDa (Fig. 2).

![Fig.2 SDS Page of the Crab Sample](image)

**DISCUSSION**
Endogenous antimicrobial peptides in marine invertebrates are exciting candidates for the development of new antimicrobial compounds, due to their broad activity spectrum and the difficulty for bacteria to develop resistance to them [14]. In the present study, hemolymph of
the crab *L. rubromaculata* 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 [15-24]. But antimicrobial activity of the present study reported crabs are not reported earlier.

Crabs are the very good resource of antimicrobial proteins with wide range of antimicrobial properties which is highly supported in the hemolymph study of *C. lucifera* [16]. In the present investigation the percentage of protein concentration of hemolymph the crab *L. rubromaculata* was recorded as 1.62 mg/ml in male and in 1.5 mg/ml female. Similar type of study was carried out in various brachyuran crabs viz., *S. serrata* and *S. tranquebarica* followed by *Nanoepisesarma minutum, Neoeipisesarma tetragonus, Metagrapes maculates, Macrothalamus depressus, T. crenta, Charybdis ferriata, C. lucifera, C. aboinsis, C. natator, Portunus pelagicus* reported in *T. crenta*. [16]. Nogaand Nagai [25, 26] reported the links between the clotting cascade and hemocyanin- based phenoloxidase systems in vitro is not clear, these results suggest that hemocyanin exists abundantly in hemolymph plasma and that it may participate in the innate immune system of the horseshoe crab.

The antioxidant activity assays deal with the kinetics of a reaction and measure the reaction rate, while antioxidant capacity assays mainly concentrated on the thermodynamic conversion and measure the number of electrons or radicals donated respectively by a given antioxidant molecules [27]. Diphenylpicrylhydrazyl (DPPH) is stable nitrogen centered free radical which can be effectively scavenged by antioxidants [28]. DPPH is also considered as a good kinetic model for peroxy radicals [29]. The ability of protein to scavenge DPPH radical was determined by the decrease in it is absorbance in spectrophotometer. When the solution of diphenylpicrylhydrazyl is mixed with that of a substance that can donate a hydrogen atom then this give rise to the reduced form (Diphenylpicrylhydrazine) which indicates the loss of this violet color [30]. The present investigation shown that the protein from *L. rubromaculata* crab Hemolymph exhibited DPPH scavenging activity. Since the effect of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability. In the present investigation purified hemolymph that showed antibacterial activity was subjected to SDS-PAGE to estimate the number and molecular weight of proteins present. After electrophoresis clear bands were detected in the gel which represented proteins of molecular weight 20-56 kDa which is similar to the antibacterial peptides in the hemolymph of the range of 1-100 kDa from *C. sapidus* [31]. Schaggerexplained tricine sodium dodecyl-sulphate polyacrylamide
gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa which fits well with range of antibacterial peptides\textsuperscript{[32]}. Okino also isolated similar molecular weight proteins from horseshoe crab hemocytes, is involved in the internalization of LPS \textsuperscript{[33]}. The hemolymph antimicrobial peptide of \textit{Thalamita crenata} was found to be 56.8kDa \textsuperscript{[16]}. The antimicrobial and antioxidant assays done so far will serve as a baseline data for further studies that may confirm the hypothesis that brachyuran crabs hemolymph are indeed potential source of novel compounds with biological potential.

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**REFERENCE**


