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Case Study

ANTIMICROBIAL PEPTIDE FROM CRAB HAEMOLYPMH OF *OCYPODA MACROCERA* (Milne Edwards 1852) WITH REFERENCE TO ANTIOXIDANT: A CASE STUDY

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ABSTRACT

Objective: To evaluate the Antimicrobial Peptide from *Ocypoda macrocera* crab haemolymph with reference to antioxidant activity.

Methods: the crude and fractionated haemolymph protein was estimated, conformed by TLC, FT-IR, and tested for antimicrobial activity. *In-vitro* antioxidant activity were determined by total antioxidant activity, DPPH, reducing power, nitrous oxide scavenging, hydrogen peroxide scavenging and ferrous ion chelating from active fraction of haemolymph.

Results: A protein was estimated from the *O. macrocera* crab haemolymph (precipitated by ammonium sulphate) which shows 15.43 to 60.34KDa. The estimated haemolymph protein was purified and detected by using thin layer chromatography and antibacterial activity of purified fraction obtained maximum zone of inhibition in the haemolymph 6th fraction (14mm). The active 6th fraction exhibit the antioxidant activity in the terms of total antioxidant activity (176 µg/ml) , dpph assay (96.11%), total reducing power (34 µg/ml), nitrous oxide scavenging (64.5%), hydrogen peroxide scavenging (91.68%) and ferrous ion chelating assay (78.54%) which has tentatively identified as amide I, II and III by FTIR spectroscopy.

Conclusion: Hence, the present study revealed that peptides from crab haemolymph have been used as an accessible source of natural antioxidants with consequent health benefits.

Keywords: *Ocypoda macrocera,* Antimicrobial peptide (AMP), Antioxidant activity

INTRODUCTION

Antimicrobial peptides (AMPs) are the major component of the innate immune system of many organisms and play an important role in protecting the host species from microbial invasion like crustaceans [1]. AMPs discovered so far have been divided into several groups based on their length, secondary and tertiary structure and presence or absence of disulfide bridges [2]. The haemolymph of crustacean have potent antimicrobial peptide showed diverse array against several human pathogens [3], which play a role in host defense response including self or non-self recognition, cell-to-cell communication, superoxide anion activity, melanisation, phagocytosis, cytotoxicity and encapsulation [4]. In recent years, natural products from marine samples have a wide spectrum of biological activities, numerous therapeutic applications include antiviral, antibacterial, antitumor activity, and very different kinds of substances have been obtained. Cyclic and linear peptides discovered from marine animals have increased our knowledge about new potent cytotoxic, antimicrobial, ion channels specific blockers, and many other properties with novel chemical structures associated to original mechanisms of pharmacological activity [5]. There is an increasing interest in antioxidants, particularly in those of free radicals in various diseases. These pathological and clinical backgrounds have prompted to investigate novel and potent antioxidant peptides from crab which are ultimately of therapeutic use. Hence, the present study focused on identify the bioactive peptides from *Ocypoda macrocera* crab from the Vellar estuary, Tamilnadu to explore it antimicrobial and antioxidant activity.

MATERIALS AND METHODS

Sample collection

The Healthy crabs collected from Annankoil landing centre located at 11° 31' N; 79° 44'E at different stages of development were used throughout the experimental period, and crabs are subjected to a single bleed collection. 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, then the sample was centrifuged at 10000 rpm for 10 min at 4°C to remove haemocytes from the hemolymph. The collected supernatant was stored at 4°C and tested within 16h [6].

Peptide purification and precipitation

The protein from the crude haemolymph sample was precipitated by ammonium sulphate (75%) and stored at 4ºC overnight. The precipitate was collected by the centrifuge at 15000 rpm for 20 min at 4ºC and pellet was resuspended in 50mM acetate buffer (pH 5). Further precipitated sample was purified by Sephadex G-50 gel column chromatography eluted with phosphate buffer and the fractions were stored at 4ºC until use [7].

Protein estimation technique

The protein content of crude, precipitated, and purified haemolymph samples were estimated by Lowry's method using Bovine serum albumin as a standard [8].

Protein separation and molecular weight determination

The proteins were separated by Sephadex G-50 was used to fractionate the precipitated haemolymph sample followed by Andrews [9] method which was eluted using PBS with flow rate of 2ml for 30min at 40˚C. SDS-PAGE and size of polypeptide chains of the given crude haemolymph protein were determined by comparing its electrophoretic mobility in SDS-PAGE gel with mobility of marker proteins of known molecular weight. SDS-PAGE was performed in 10% separating gels, 5% stacking gel according to the method described by Laemmli *et al.*[10]

Characterization of protein by thin layer chromatography and FT-IR spectroscopy:

Thin layer chromatography was performed on Butanol, Acetic acid, and water in the ratio of 4:1:5. The 10 µl of each fraction was spotted in the silica coated TLC plate and run in closed chamber previously equilibrated with solvent. Then the TLC plates were taken and solvent front was marked. The slides were air dried and sprayed with 0.1% ninhydrin, for detecting the compounds [11].

The lyophilized active fraction sample was subjected to FT-IR analysis. The IR spectrum of the protein was recorded with a Perkin-Elmer model 297IR spectrophotometer. One part of the extract was mixed

with 99 part of dried potassium bromide and it was scanned between 600–4000 wave number (cm^{-1}) at a speed of 1 micron/ min and with a programmed slit opening and air as reference.

Antimicrobial assay

Antimicrobial activity of haemolymph of *Ocypoda macrocera* against eight bacterial strains viz. *Salmonella paratyphii*, *S. typhi*, *Proteous mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Escherichia coli* and *Klebsiella oxytoca* and four fungal pathogens viz. *Aspergillus niger, A. flavus, Candida albicans* and *Penicillium citrinum* were obtained from the department of Medical Microbiology (Raja Muthiah Medical College Hospital) Annamalai University, Annamalai Nagar, Chidambaram.

In-vitro antibacterial assay was carried out by disc diffusion method[12]. Filter paper (Whatmann No. 1) discs with 4mm diameter were impregnated with known amount test samples of the crude, ammonium sulphate precipitated crab haemolymph, column purified active fraction, and positive control contained a standard (Streptomycin) antibiotic disc. The impregnated discs along with control (incorporated with sterile water) were kept at the center of Nutrient Agar Plates, seeded with test bacterial cultures. After incubation at room temperature (37°C) for 24 hrs antibacterial activity expressed in terms of diameter of zone of inhibition was measured and recorded.

In-vitro antifungal activity was determined using the technique of Mercan et al.,[12]. Four different species of fungal pathogen was inoculated by spread plate method using 0.1 ml of 72 hrs old culture, maintained in mycological broth. Filter paper (Whatmann No.1) discs with 4mm impregnated with test samples of the crude, ammonium sulphate precipitated crab haemolymph, column purified active fraction and positive control contained 300 µg of standard (Nystatin) antibiotics. Control discs were placed (solvent impregnated) on the PDA plates. After incubation at 30°C for 5-7 days, antifungal activity was measured in term of diameter of zone (including the disc within) in mm and recorded.

Antioxidant activity

The antioxidant activity of the protein was evaluated in terms of total antioxidant activity [13] dpph assay [14] total reducing power [15] hydrogen peroxide scavenging [16] nitrous oxide scavenging activity [17] and Ferrous ion chelating assay[18].

RESULTS

Protein purification and estimation

The protein content of cell free haemolymph of crab species *Ocypoda macrocera* was 872 µg/ml and ammonium sulphate precipitated haemolymph showed 672 µg/ml of protein (Figure 1). The precipitated haemolymph was purified through the Sephadex G-50 gel permeation chromatography, the fractions are lyophilized which protein estimation is done. A totally eight fractions were collected, among these fractions, a significant protein content was showed high in 6th fraction (357 µg/ml) compare than the other fractions.

Molecular weight determination

The haemolymph sample was subjected to SDS polyacrylamide gel electrophoresis to estimate the molecular weight of proteins. SDS-PAGE of partial purified ammonium sulphate precipitated haemolymph of crab species *Ocypoda macrocera* showed 15.43 to 60.34KDa (Figure 2).

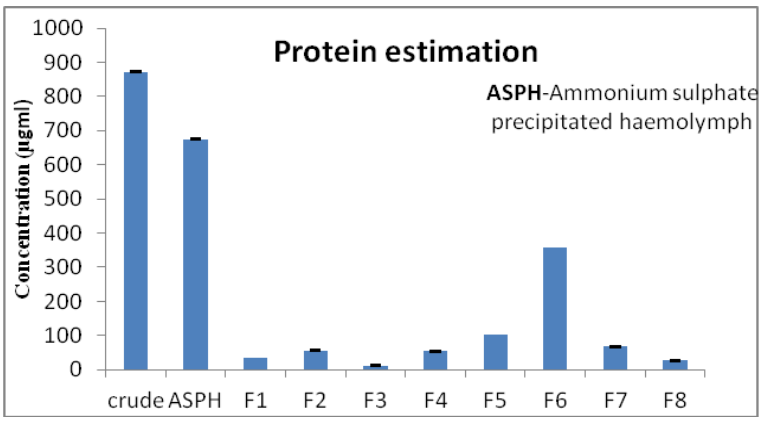

Fig. 1: Protein estimation of crude, Precipitated and purified active fraction hameolymph from *O. macrocera*

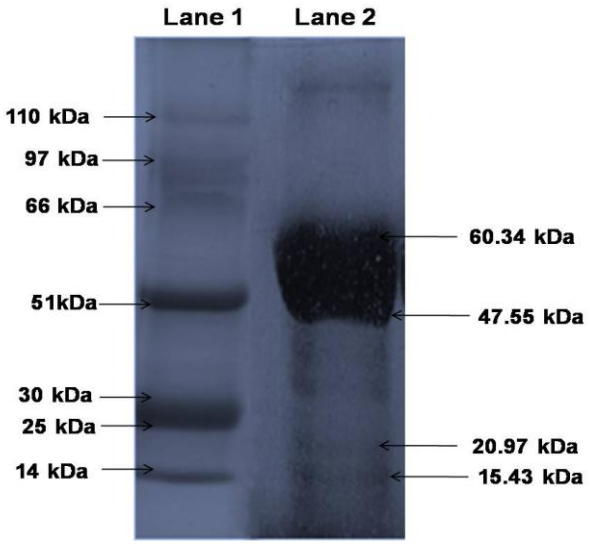

Fig. 2: Electrophoretic separations (SDS-PAGE) of haemolymph of *O. macrocera*

Lane 1-Marker; **lane 2-** Ammonium sulphate precipitated haemolymph

Characterization of protein by thin layer chromatography and FT-IR spectroscopy

The active fraction was subjected to TLC studies. After spotting the fraction on TLC plate and spraying with ninhydrin, pink spots were observed. As it was well known that peptides are known to yield pink spots with ninhydrin and since it had yielded pink spots it was taken up for further investigation leading to the isolation of peptides. The presence of ninhydrin positive spots, indicated that the possibility of peptides (Figure 3). Infrared spectroscopy of the bioactive fraction was recorded using Bio-Rad FTES-40 equipment. The bioactive fraction spectra showed 21 peaks.

Table 1: Purified active fraction of the peptide samples in FTIR

Purified active fraction (6th) sample	
Band position	**Assignment**
3429	NH stretch, coupled with HI
3217	CH2 assymmetrical stretch
2918	CH2 assymmetrical stretch
2849	CH2 assymmetrical stretch
1652	C=O stretch
1635	$CN_3H_5^+$
1568 – 1559	NH bend coupled with CN stretch
1405	COH
1245	CH
1125 – 1021	C-O
929 – 621	Skeletal stretch

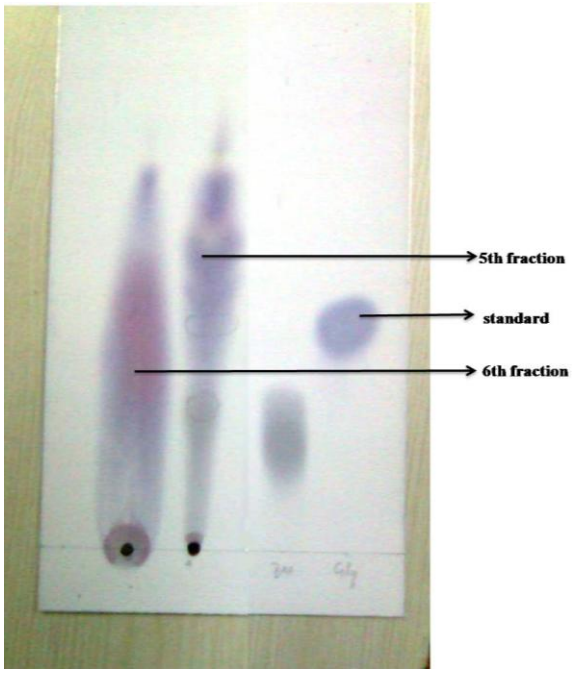

Fig. 3: TLC plate represented the presence of amide groups in active fractions.

The FT-IR spectrum of peptide revealed that the peak 3429 indicates the presence of NH stretching coupled with HI. Likewise 1568 – 1559 indicates the bending of NH coupled with CN stretch. The wave number 3217, 2918 and 2849 characteristic of asymmetrical stretching of CH_2, whereas 1652, 1635, 1405, 1245, 1125-1021 and 929-621 positions of the spectrums are the characteristic C=O stretching, $CN_3H_5^+$, COH, CH, C-O and Skeletal stretch respectively. These asymmetrical stretching, bending and skeletal stretch indicated that the presence of the amide groups (Table 1, 2 & Figure 4).

Table 2: Represented functional groups of active fraction of purified haemolymph sample in FT-IR analysis.

Band position (cm^{-1})	**Functional Groups**
3217 cm^{-1}	Amide A
2918 - 2849 cm^{-1}	Amide B
1652 cm^{-1}	Amide I(α- helical)
1645 cm^{-1}	Amide I(Random coil)
1635 cm^{-1}	Amide I(β-sheet)
1568 - 1559 cm^{-1}	Amide II
1405 – 1245 cm^{-1}	Amide III

Among the peaks, ten were found to be prominent and were representing amide groups (Amide I - 1652cm^{-1} to 1635cm^{-1}, Amide II 1568cm^{-1} - 1405cm^{-1}, Amide III 1245cm^{-1} to 1021 cm^{-1}, Amide IV 667cm^{-1} to 621cm^{-1}, Table 2). Amide II (1045cm^{-1}) peak was maximum absorption which implies the presence of the protein/peptide in the bioactive 6th fraction.

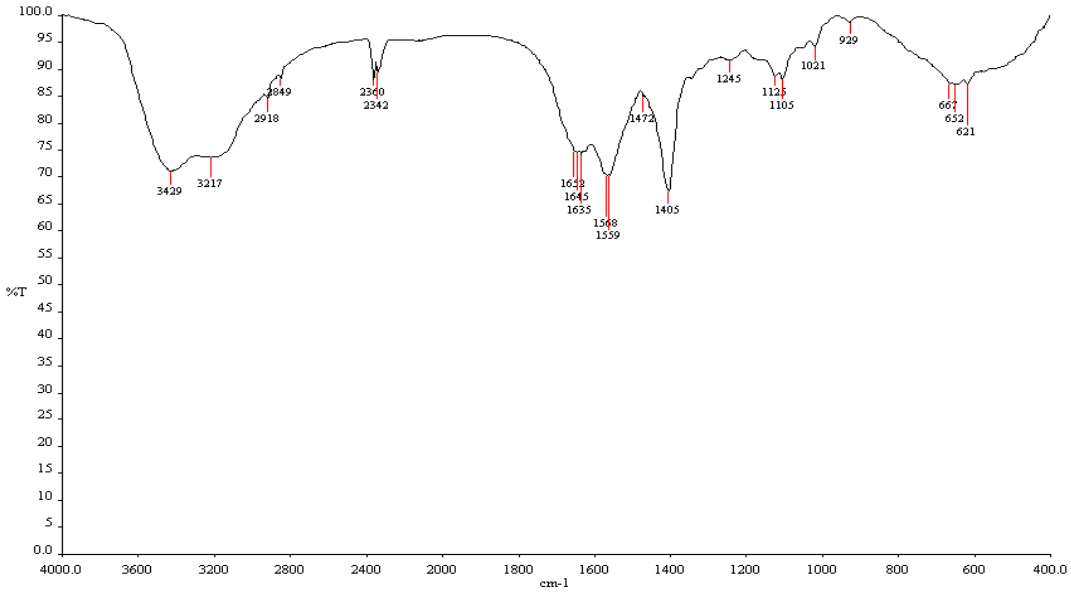

Fig. 4: FT-IR- spectra represented to partially purified protein group in (6th) active fraction of *O. macrocera.*

Antimicrobial activity

In the present study antimicrobial activity also conducted after the confirmation of proteins from crab haemolymph. The crude, ammonium precipitated and purified haemolymph of the *O. macrocera* was evaluated against eight different strains of gram positive (2) and gram negative bacteria (6), fungal human pathogens to investigate the presence of the antimicrobial activity.

In antibacterial activity of *O. macrocera* zone inhibition varied from 3 to 14 mm. Maximum diameters was noted against *Klebsiella oxytoca* (14mm) and minimum zone of inhibition (3) was recorded against *Pseudomonas aeruginosa* (7mm) followed by *K. pneumonia* (9mm), *Salmonella paratyphii* (10mm) and *S. typhii* (10mm), *Proteous mirabilis* (12mm), *E. coli* (12mm) and *Staphylococcus aureus* (12mm). Likewise, maximum inhibition zone (21mm) was recorded in ammonium sulphate precipitated haemolymph of *Ocypoda macrocera* and minimum zone of inhibition (9mm) was observed against *Pseudomonas aeruginosa*. Figure 5 showed positive activity of all eight pathogenic strains.

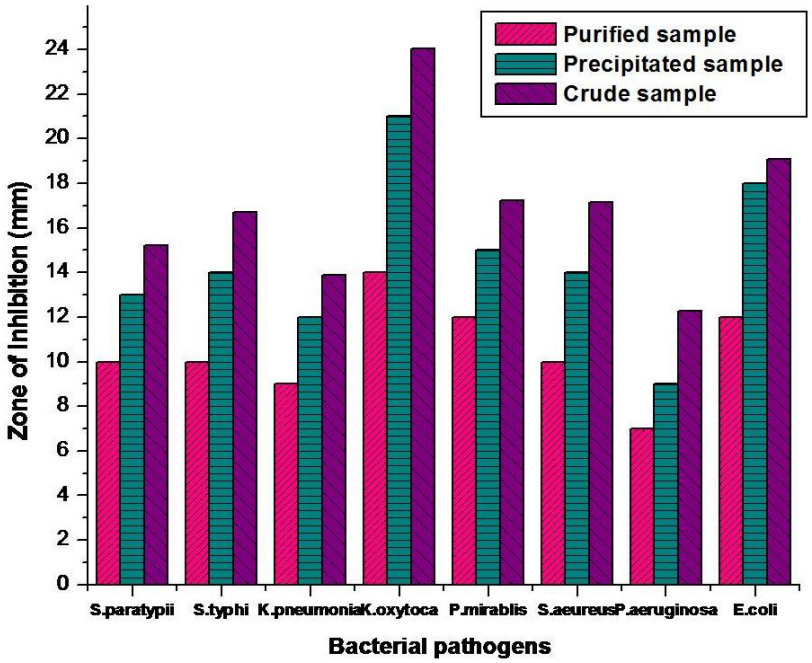

Fig. 5: Antibacterial activity of haemolymph *O. macrocera*

In antifungal activity zone of inhibition of 12 mm was observed using crude haemolymph of *O. macrocera* against *Aspergillus flavus* followed by *Penicillium citrinum* and *A. niger*. Minimum inhibition was observed against *Candida albicans* (5mm). Whereas highest zone of inhibition was observed in the ammonium precipitated haemolymph of *O. macrocera* against *A. flavus* (19mm) followed by *A. niger*. Minimum inhibition zone was noted against *C. albicans* (10mm) (Figure 6). Haemolymph of *O. macrocera* showed moderate antifungal activity against all four fungal pathogens and all are susceptible to both crude and precipitated samples.

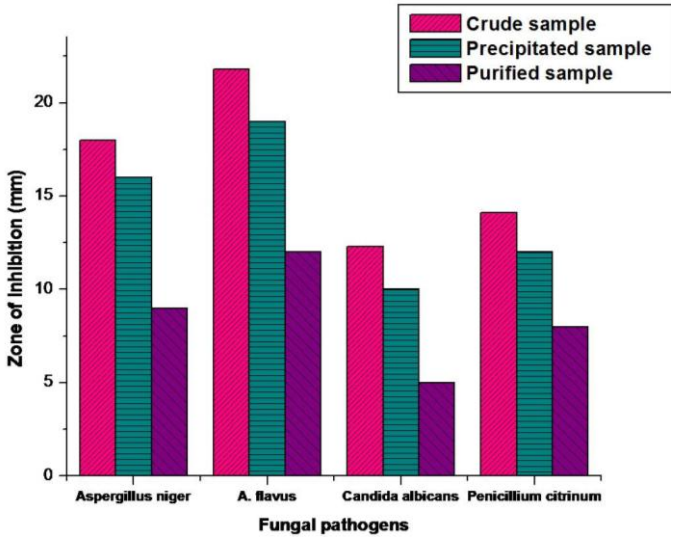

Fig. 6: Antifungal activity of haemolymph *O. macrocera*

Screening for the antioxidant activity of active 6th fraction

The antioxidant activity of purified active protein from the *O. macrocera* haemolymph were measured in different system of assay such as Total antioxidant activity, DPPH assay Ferrous ion chelating activity, H_2O_2 scavenging activity, NO scavenging activity and reducing power assay. The protein was found to have different levels of antioxidant activity in different concentration (20, 40, 60, 80 & 100 µg/ml) tested. IC_{50} values were calculated.

Total antioxidant activity

The Total antioxidant activity of protein of *O. macrocera* crab was assessed by the formation of green phosphate complex at acidic pH. Figure 7 illustrate that the antioxidant capacity of the protein (20-100 µg/ml) with standard ascorbic acid. The result shows that purified fraction had the potential activity. This indicates that the partial purified protein of crab haemolymph sample has a good source of natural antioxidants.

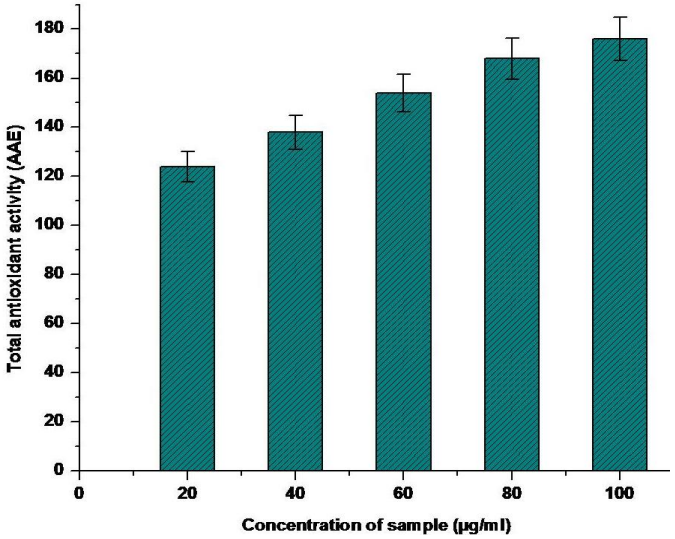

Fig. 7: Total antioxidant activity of peptides (20 - 100 µg/ml), expressed in AAE

DPPH assay

The free radical scavenging activity of protein from crab *O. macrocera* haemolymph was assessed by the DPPH assay. Figure 8 shows that a significant decrease in the concentration of DPPH radical due to scavenging ability of the protein. The result shows that purified 6th fraction from crab had the significant DPPH scavenging activity (96.11%) at 100µg/ml.

Total Reducing power

Figure 9 shows that the reducing capacity of *O. macrocera* haemolymph protein compared to standard EDTA. Purified protein fraction (100µg/ml) showed higher reducing ability when compared with the control (0.817).

H_2O_2 Scavenging activity

The scavenging effect of hydroxyl radical was investigated using the fenton reaction and the results were shown as an inhibition rate in figure 10. The purified fraction of *O. macrocera* crab haemolymph exhibited the highest inhibition of about 91.68 % at the concentration of 100µg/ml

Nitrous oxide radical scavenging activity

Suppression of Nitric oxide free radical release may be attributed to a direct nitric oxide free radical scavenging effect to decrease the amount of nitrite generated from the decomposition of sodium nitroprusside. The result shows that purified fraction of haemolymph protein had scavenging activity 64.5% at the concentration of 100µg/ml compared with the standard Ascorbic acid (Figure 11).

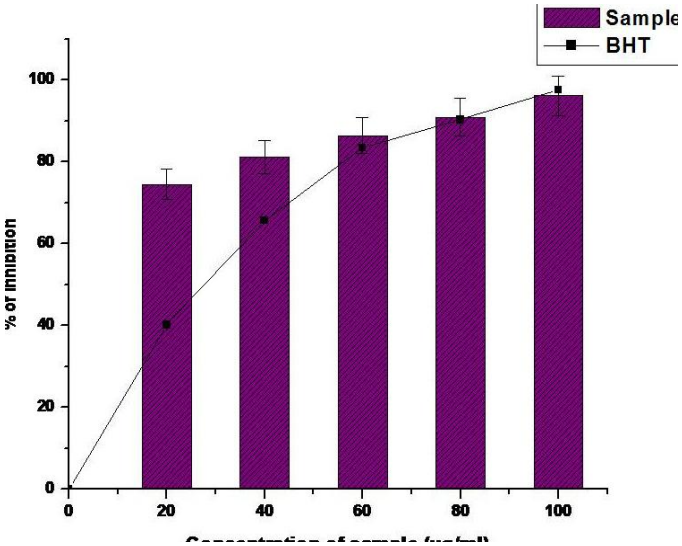

Fig. 8: DPPH scavenging activity of peptide (20 - 100 µg/ml), compared with standard BHT

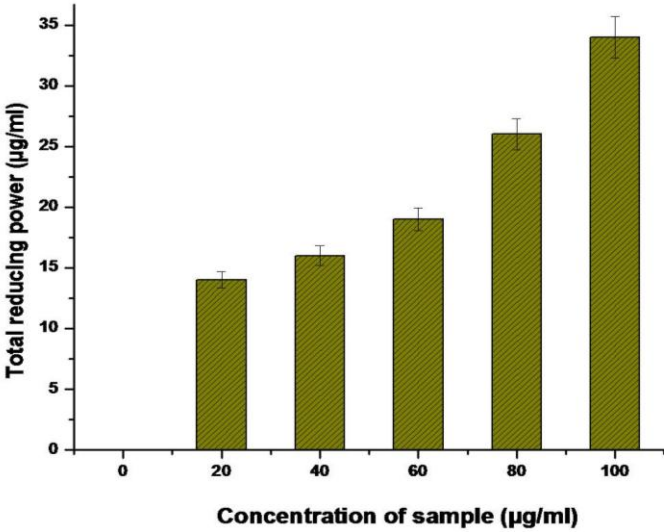

Fig. 9: Total reducing power of peptide (20 - 100 µg/ml) compared with standard Ascorbic acid.

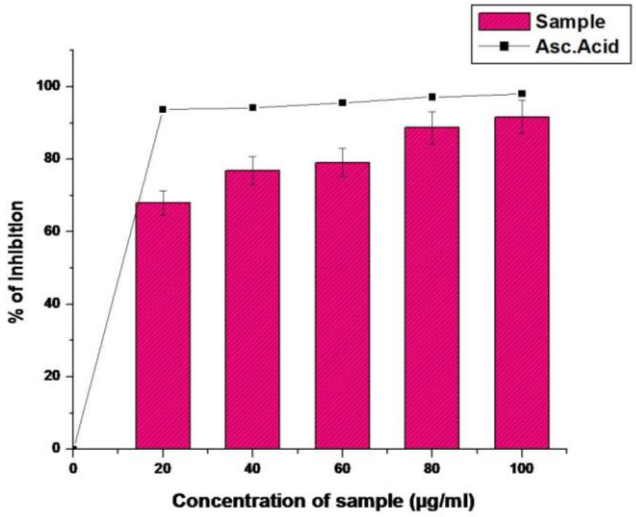

Fig. 10: Hydrogen peroxide scavenging activity of peptides (20 - 100 µg/ml), compared with standard Ascorbic acid

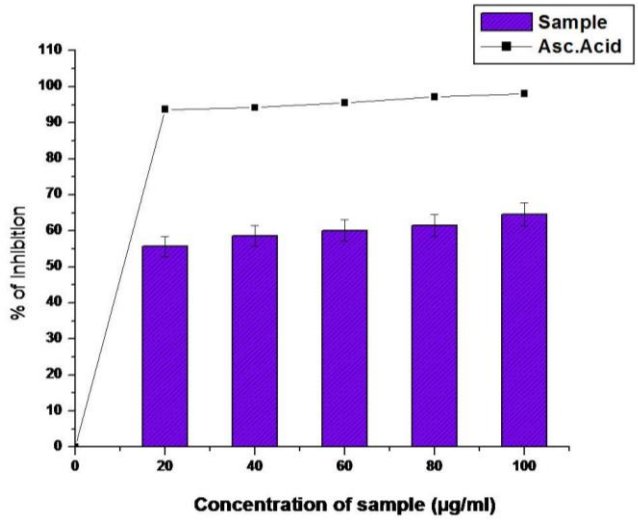

Fig. 11: Nitrous oxide scavenging activity of peptides (20 - 100 µg/ml), compared with standard Ascorbic acid

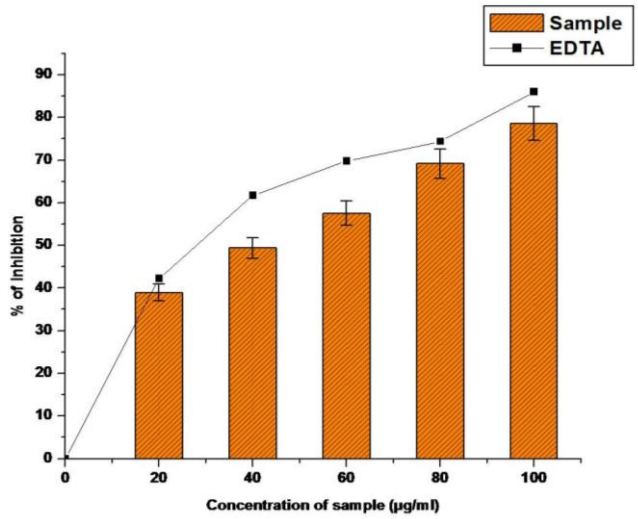

Fig. 12: Ferrous iron chelating activity of peptides (20 - 100 µg/ml), compared with standard EDTA

Ferrous ions - chelating ability

The chelating ability of ferrous ion scavenging activity (Figure 12) shows that a potential of ferrous ion chelating activity of purified fraction of the protein from haemolymph of the *O. macrocera*. The chelating abilities of purified fraction from haemolymph protein on ferrous ion were potential at various concentrations (20-100 µg/ml) which exhibit 38.92%, 49.37%, 57.54%, 69.16% and 78.54% respectively.

DISCUSSION

In the present study, *Ocypoda macrocera* crab haemolymph peptide was extracted and purified product obtained from eight fractions. Among these, 6th fraction of the precipitated peptide from hemolymph showed significant antibacterial 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[19] *Thalamita crenata* [20] and *Charybdis lucifera* [21]. [22] reported that crude root extract of *Rauwolfia serpentina* showed an efficient activity of 22.5 ± 2.5 mm against *S. typhii* bacteria. In the present study clearly shows that the hemolymph of *O. macrocera* crab exhibit significant activity and more detailed studies might lead to novel structures with promising potential as antimicrobial agents.

In present study, the molecular weight determination of partial purified (ammonium precipitated) haemolymph sample range from15.43 to 60.34KDa. A similar molecular weight protein was also isolated by *S. serrata*. Schagger and Jagow [23] explained tricine Sodium dodecyl – sulphate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100kDa which fits well with a range of antibacterial peptides. Okino *et al.* [24] also isolated similar molecular weight proteins from the horseshoe crab hemocytes. A haemolymph protein of 47kDa named hemolin, composed of repeated immunoglobulin domains, thought to have a role in immune recognition and in modulation of defensive responses in *Hyalophora cecropia* and *Manduca sexta* [25]. Comparative relationships between similar haemolymph proteins and developing crab ovaries have been reported previously. Bedford and Reid [26] suggest that Polyacrylamide gel electrophoresis can be used as the crystalline style of molluscs. 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 haemolymph of the range of 1-100 kDa from *C. sapidus*[27]. Hoq *et al.*, [28] isolated and characterize the antibacterial peptides from mud crab, similarly Rameshkumar *et al.,*[20] antimicrobial peptide of *T.crenata* haemolymph were isolated and determined the molecular weight of the peptides (56.8kDa)

Antioxidant activity

In 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 [29]. The total antioxidant activity assay quantifies the combined action of all antioxidant present in the sample capable of reduction of Mo (VI) to mo (V) and subsequently formation of a stable green phosphate [Mo (V)] complex at acidic pH [30]. Through the formation of green color indicate the antioxidant activity of peptide from haemolymph of crab *Ocypoda macrocera.*

Diphenylpicrylhydrazyl (DPPH) is stable nitrogen centered free radical which can be effectively scavenged by antioxidants [31]. DPPH is also considered as a good kinetic model for peroxylradicals [32]. The ability of protein to scavenge DPPH radical was determined by the decrease in it is absorbance in spectrophotometer. When, the solution of diphenylpicrylhydrazyl was 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 [33]. The present, investigation shown that the partial purified protein from *O. macrocera* crab haemolymph exhibited DPPH scavenging activity. Since the effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability.

The reducing power ability of partial purified protein of *O. macrocera* crab haemolymph greatly depends on the presence of reductones, which have exhibit antioxidant potential by breaking the free radical chain by donating a hydrogen atom [34]. Hydrogen peroxide is a weak oxidizing agent[15] and once inside the cell it can probably react with Fe^{2+} and possibly Cu^{2+} to form hydroxyl radicals and this may be the origin of toxic effects [35]. The result of present study reveals that there is a strongest H_2O_2 scavenging activity was observed for protein at various concentrations when compared to be good scavenger of hydrogen peroxide. But the maximum activity has observed in *O. macrocera* crab hemolymph protein can be a good antioxidant for removing hydrogen peroxide free radicals.

When the nitrous oxide radicals react with oxygen free radicals ($O_2^{\bullet-}$) to form peroxynitrate which damages biomolecules like lipids and nucleic acids and toxicity also multiplies. Nitric oxide free radicals generated from the sodium nitroprusside react with oxygen to form nitrate [15]. In the present study, protein of the crab haemolymph at the concentration from 20 to 100µg/ml exhibited 55.68%, 58.64%, 60.11%, 61.5% and 64.5% respectively. The present result suggests that the crab *O. macrocera* haemolymph protein might be potent agent for scavenging of Nitric oxide. In the present study protein of the *O. macrocera* crab haemolymph at the various concentrations showed higher absorbance to indicating the proteins are the best source of antioxidant compounds.

The chelating effects of various extracts on Fe^{2+} were determined by the formation of ferrozine -Fe^{2+} complexes. Chelating agents are able to capture ferrous ion before ferrozine, thus hindering the formation of ferrozine -Fe^{2+}. Spectrophotometic examination of ferrozine-Fe^{2+} absorbance can accordingly be used to calculate ferrous ion chelating activity. The metal chelating capacity is important since it reduces the concentration of transition metals that may act as catalysts to generate the first few radicals to initiate the radical-mediated oxidative chain reactions in biological and food systems. Ion chelating agents also may inhibit the Fenton reaction and hydro peroxide decomposition [36]. Through the formation of ferrozine complex indicate the ferrous ion chelating ability of peptides from haemolymph of *O. macrocera.*

CONCLUSION

In present study, antimicrobial activities of the crude, precipitated and purified peptides and antioxidant activity of purified protein fraction from haemolymph of *Ocypoda macrocera* were investigated. The peptides were found to possess antimicrobial activity, radical scavenging and antioxidant activities, as determined by scavenging effect on the total antioxidant activity, DPPH, chelating effect on ferrous ions, Hydrogen peroxide scavenging activity, Nitric oxide scavenging activity and reducing power. In this study, it is found that the peptides from *O. macrocera* have antioxidant activity and antimicrobial activity. Thus it can be concluded that the peptides from *O. macrocera* crab haemolymph can be used as an accessible source of natural antimicrobial and antioxidants with consequent health benefits.

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