

Multifunctional roles of hemolymph serum in the innate immunity of freshwater crab *Barytelphusa cunicularis* (Westwood in Sykes, 1836)

Sundararajan Sowmiya¹, Rangasamy Shanthi^{1*}, Mullaivanam Ramasamy Sivakumar² and Francis Abisha Adline¹

¹Laboratory of Crustacean Biology, University of Madras, Guindy Campus, Chennai, Tamil Nadu, India

²Weevil Biotech Pvt. Ltd, No. 30, Bharathi Colony Annex, Kaikankuppam, Valasaravakkam, Chennai 600087, Tamil Nadu, India

Received 9 October 2025; revised 19 January 2026

Understanding innate immunity in crustaceans is essential due to their ecological and aquaculture importance, yet the functional roles of hemolymph serum in freshwater crabs remain poorly characterized. In this study, the immune properties of serum from the freshwater crab *Barytelphusa cunicularis* were investigated with emphasis on its biochemical composition and functional activity. Biochemical profiling revealed a predominance of proteins over carbohydrates and lipids, indicating a serum rich in effector molecules. Phenoloxidase (PO) activity, a key enzyme in invertebrate immunity, exhibited optimal activity at 25 °C in Tris-HCl buffer (pH 7.5) using DL-DOPA as substrate, and was significantly influenced by immune-related activators and inhibitors such as trypsin, SDS, laminarin, and PTU ($P < 0.05$). Hemagglutination assays revealed lectin-like activity, with the serum strongly agglutinating vertebrate erythrocytes, while carbohydrate inhibition confirmed galactose-specific binding. Additionally, the serum exhibited pronounced lysozyme and antimicrobial activities against five bacterial and three fungal pathogens. Microbial agglutination titers (32–512) further demonstrated its capacity for broad pathogen recognition and binding. These findings highlight the coordinated immune strategy of *Barytelphusa cunicularis* serum involving pathogen recognition, enzyme activation, and antimicrobial defense, providing new insights into the multifunctional roles of hemolymph serum in crustacean innate immunity.

Keywords: Phenoloxidase, Hemagglutination, Lysozyme, Microbial agglutination, Antimicrobial defense

Introduction

Aquaculture is a major contributor to global food production, with Asian countries leading at 70%, followed by America, Europe and Africa. The industry is evolving through improved practices, resulting in economic and nutritional benefits¹. Crustaceans form a significant component of this sector, contributing 15,366 kilotons to global fisheries and aquaculture production². Among them, freshwater crabs represent a notable subset of Brachyuran species and are recognized for their ecological and medicinal importance. Freshwater crustaceans contribute substantially to inland aquaculture, with increasing cultivation and yield reported in recent years due to their nutritional value and market demand. However, disease outbreaks continue to limit productivity, highlighting the need to understand innate immune mechanisms to support sustainable freshwater crab culture^{3,4}. *Barytelphusa cunicularis*, widely distributed in freshwater rivers and wetlands across

India, plays a key ecological role as a detritivore and contributes to local fisheries, providing an important source of protein and income. These characteristics make it an ideal species for studying crustacean immunity⁵. Their high protein content, quality meat, unique flavour, and mineral richness have gained increasing attention in international markets⁶. Although crabs contain relatively low carbohydrate levels, they are valued for their energy content derived primarily from proteins and lipids⁷. Fatty acid profiling remains crucial for nutritional assessment and in crustaceans, lipid metabolism is largely regulated in the ovaries and hepatopancreas, especially during fasting periods⁸. Despite diverse studies on crab immunity, bacterial, shell-related, *Aeromonas* and viral diseases still threaten their mass culture and production. Understanding the immune mechanisms of freshwater crabs is essential for sustaining aquaculture productivity, as robust immunity helps prevent disease outbreaks and reduces mortality in mass culture systems⁹⁻¹¹. Decapod crustaceans, including crabs, lack adaptive immunity and instead depend solely on an innate immune system comprising cellular and humoral

*Correspondence:

Phone: +91 9585980194

E-mail: drshanthisarojini@gmail.com

components¹²⁻¹⁴. Cellular immunity involves processes such as phagocytosis, encapsulation, nodule formation, and pathogen lysis mediated by different hemocyte types^{15,16}. Similarly, the humoral immune mechanism of crustaceans mainly comprises of active immune molecules present in the serum, plasma and hemocyanin^{9,14,17} which includes agglutinin or lectin¹⁸, antimicrobial proteins¹⁹, prophenoloxidase (proPO) system^{20,14}, haemolytic molecules²¹ lipopolysaccharide (LPS) binding proteins²² and anticancerous agents²³.

Among these, melanization mediated by phenoloxidase (PO) activation is a key immune response in crustaceans^{9,10,24}. In crustaceans, proPO activating enzyme and its components are located in the granules of hemocytes and released by pattern recognition due to tissue damage or danger signalling²⁵. Recently, many proteinases and regulatory proteins are found to be involved in initiating proPO cascade resulting in melanization and activation of tolls²⁶. However, phenoloxidase (PO) is copper dependent and the inactive proPO gets activated to PO by proteolytic reactions. PO oxidises *o*-diphenols into *o*-quinones which polymerizes to form melanin by non-enzymatic polymerization reaction^{14,17}. Additionally, PO catalyses the conversion of tyrosine to DOPA and subsequently to DOPA-quinone, a melanin precursor, emerging evidence suggests that a caspase-1-like enzyme may modulate PO activity by cleaving proPO into fragments, limiting melanization but enhancing microbial agglutination²⁷. Like PO system, Lectins, or agglutinins, are another key group of immune molecules in crustaceans. These carbohydrate-binding proteins recognize glycan structures such as glycolipids, glycoproteins, monosaccharides and oligosaccharides²⁸. Found ubiquitously in nature, lectins are categorized as plant, animal or microbial²⁹. In crustaceans, lectins exhibit species-specific structures with one or more carbohydrate recognition domains (CRDs)³⁰. Crustacean lectins are classified based on their structure and function such as C-type, P-type, M-type, L-type, F-type, I-type, R-type, X-type, galectins, fibrinogen-like domain lectins, chitinase like lectin, ficolin and calnexin or calreticulin³¹. Among these, calcium-dependent C-type lectins are the most studied in crustaceans due to their diverse immune functions including antibacterial activity, regulation of AMPs and proPO activation, enhancement of phagocytosis^{32,33,34}. Lysozymes represent another evolutionarily conserved class of defense enzymes in crustaceans³⁵. These enzymes hydrolyze the peptidoglycan layer of bacterial cell walls,

leading to cell lysis. In addition to their antimicrobial roles, lysozymes exhibit antiviral, anti-inflammatory, antioxidant, and anticancer properties^{15,36}.

Antimicrobial peptides (AMPs) represent another vital class of humoral immune effectors in crustaceans, acting as a first line of defense against diverse pathogens. These small, cationic peptides, (<10 kDa) specifically target the negatively charged membranes of microorganisms^{37,38}. They are released by specific hemocytes into the hemolymph upon recognition of microbial stimuli³⁹. Crustacean AMPs, such as crustins, are multifunctional, contributing to both immunity and physiological regulation⁴⁰. In crabs, AMPs include arasins⁴¹, panusins (β -defensin-like peptides)⁴² and scyreprocin⁴³ highlighting the structural and functional diversity of these effectors. The freshwater crab *Barytelphusa cunicularis* is an ecologically important yet underexplored species with promising nutritional and biomedical potential. Phenoloxidase, lectins, lysozymes, and antimicrobial peptides were selected as key humoral components because they represent major effectors of crustacean innate immunity, mediating pathogen recognition, microbial killing and immune response^{44,45}. The present study aimed to characterize the hemolymph serum of *B. cunicularis* through biochemical and immunological analyses, including phenoloxidase activity and its modulation, hemagglutination and sugar inhibition assays, lysozyme activity, antimicrobial screening and microbial agglutination. These findings provide new insights into the innate immune repertoire of *B. cunicularis* and enhance understanding of crustacean humoral defense mechanisms.

Materials and Methods

Collection and maintenance of animals

Adult, intermoult, uninjured crabs (n = 40), each weighing approximately 50 ± 2 g, were collected during the pre-monsoon season (February to April) from the riverine region of Tirupattur District, Tamil Nadu, India. The crabs were collected from freshwater with a pH of 7.6 and normal ambient water temperature. Both male and female crabs were included in the study. The collected animals were transported to the laboratory and maintained in aerated glass tanks (0.5 × 0.5 m). Each tank contained 5 crabs with approximately 25 L of freshwater per tank. The animals were maintained at temperatures ranging from 27 to 30 °C and were acclimatized for

7 days before the commencement of experiments. During acclimatization, the crabs were fed alternately with egg and meat and water was renewed regularly to maintain hygiene. Meanwhile, Crabs were given to Zoological Survey of India (ZSI) for identification of Genus and species.

Hemolymph collection and preparation of serum

Hemolymph was collected from the cut end of the dactyls region of the walking legs into pre-chilled centrifuge tubes held on ice. A total of approximately 80 ml of hemolymph was obtained from freshwater crab *Barytelphusa cunicularis* (WESTWOOD IN SYKES, 1836) n= 40. Samples were allowed to clot overnight at 4 °C. The clots were gently disrupted using a sterile glass rod and centrifuged at 10,000 rpm for 20 min at 4 °C. The resulting supernatant (serum) was collected and stored at -20 °C for further analysis.

Chemicals and reagents

Ethanol, orthophosphoric acid, bovine serum albumin, Coomassie Brilliant Blue G-250, anthrone, sulfuric acid, glucose, chloroform, methanol, vanillin, cholesterol, Tris(hydroxymethyl)aminomethane, tyrosine, tyramine, L-3,4-dihydroxyphenylalanine, DL-3,4-dihydroxyphenylalanine, dopamine, catechol, hydroquinone, pyrogallol, phenylthiourea, trypsin, laminarin, sodium dodecyl sulfate, Triton X-100, Alsever's solution, dextrose, sodium citrate, citric acid, sodium chloride, streptomycin, magnesium chloride, barium chloride, calcium chloride, manganese(II) chloride, and potassium chloride were used in the present study. Simple sugars, amino sugars, N-acetyl amino sugars, and glycoproteins were used as substrates for hemagglutination and related assays. *Micrococcus lysodeikticus* (ATCC No. 4698; Sigma-Aldrich) was used as the substrate for the lysozyme assay. Thiosulfate-citrate-bile salts-sucrose agar, eosin methylene blue agar, cetrinide agar, Mueller-Hinton agar, Mueller-Hinton broth and amphotericin B were used for antimicrobial assays. All chemicals and culture media were of analytical grade and procured from Sigma-Aldrich (St. Louis, MO, USA).

Biochemical analysis of serum

Biochemical parameters of the serum were assessed using standard methods: total protein content⁴⁶ total carbohydrate⁴⁷ and total lipids⁴⁸.

Phenoloxidase activity

Oxidation of phenolic substrates by serum

To assess the oxidative activity following Sivakumar *et al.*⁴⁹ 0.1 mL of serum was incubated

with 0.9 mL of various phenolic substrate solutions (Tris-HCl 50 mM, pH 7.5) containing 5 mM of tyrosine, tyramine, L-DOPA, DL-DOPA, dopamine, catechol, hydroquinone and pyrogallol. After incubating for 10 min at 25 °C the developed colour was read spectrophotometrically at 400-700 nm against a reagent blank where the specific substrates were substituted for serum. Multiple phenolic substrates were employed to evaluate substrate specificity of serum phenoloxidase and to distinguish between monophenolase and diphenolase activities; DL-DOPA was selected for subsequent optimization as a representative diphenolic substrate reflecting catechol oxidase type PO activity.

Optimal conditions of PO activity in serum

For the assessment of the serum oxidative activity towards DL-DOPA the following experimental procedure was employed. Initially, 0.1 mL of serum was incubated with 0.9 mL of DL-DOPA solution (Tris-HCl 50 mM, pH 7.5) at various concentrations ranging from 1.0 to 10.0 mM for duration of 10 min at 25 °C. The resulting colour development was observed spectrophotometrically at 455 nm against a reagent blank (DL-DOPA). All subsequent experiments followed the same procedures. Specifically, 0.1 mL of serum was incubated with 0.9 mL of a 7.5 mM DL-DOPA solution (Tris-HCl 50 mM, pH 7.5) for different time intervals from 1 to 45 min at 25 °C. To investigate the impact of the buffer's ionic strength on the serum mediated oxidation of 7.5 mM DL-DOPA, 0.1 mL of serum was incubated with 0.9 mL of DL-DOPA prepared in Tris-HCl buffer (pH 7.5) at varying ionic strengths ranging from 10 to 60 mM, all at 25 °C. Furthermore, the ability of the serum to oxidize 7.5 mM DL-DOPA was evaluated at different pH values. This involved incubating 0.1 mL of serum with 0.9 mL of the substrate solution prepared in Tris-HCl buffer (50 mM) at pH values ranging from 6.0 to 9.0 for duration of 10 min at 25 °C. Lastly, the influence of different temperatures on the oxidation process was examined. To achieve this, 0.1 mL of serum was incubated with 0.9 mL of a 7.5 mM DL-DOPA solution prepared in 50 mM Tris-HCl (pH 7.5) buffer subjecting the samples to temperatures ranging from 10 to 80 °C for duration of 10 min.

Effects of inhibitor and activators on PO activity

Phenylthiourea (PTU) was used to confirm copper-dependent phenoloxidase activity, while trypsin, SDS, Triton X-100 and laminarin were included to evaluate proteolytic, detergent-mediated and pathogen-associated

activation of the prophenoloxidase system. PTU at concentrations ranging from 1 to 10 mM was prepared and added to serum samples. As a control, Tris-HCl buffer (50 mM, pH 7.5) was substituted for PTU and pre-incubated at 25 °C for 5 min. The reaction mixtures from both the control and experimental trials were then incubated with DL-DOPA (7.5 mM) for 10 min at 25 °C.

Serum 0.1 mL was mixed separately with 0.1 mL of trypsin (1-12.5 mg.mL⁻¹), SDS (1-10 mg.mL⁻¹), Triton X-100 (1-10 mg.mL⁻¹) and laminarin (1-10 mg.mL⁻¹). As a control, Tris-HCl buffer (50 mM, pH 7.5) was used instead of these substances and pre-incubated for 5 min at 25 °C. Both the control and experimental samples were then incubated with 0.8 mL of DL-DOPA (7.5 mM) for 10 min at 25 °C. The optical density of the control and experimental samples was measured using a spectrophotometer at 455 nm, compared against a reagent blank consisting of the substrate solution (DL-DOPA).

Hemagglutination activity

Collection and preparation of erythrocytes

Blood samples were collected from mammals such as goat, sheep, chicken, ox, cow and buffalo at government slaughter house (Perambur, Chennai, India). Human blood samples (A, B and O blood groups) was obtained from healthy volunteers via venous puncture with informed consent. Samples were stored in Alsever's solution⁵⁰ at 10 °C and used immediately. Erythrocytes were washed three times in 0.9% physiological saline (350 × g, 8 min, 25 °C) and once with Tris-buffered saline (50 mM Tris, 100 mM NaCl, pH 7.5), then resuspended as a 1.5% suspension (v/v).

Hemagglutination (HA) assay

For the determination of HA activity following (Sivakumar *et al*⁴⁹) V-bottom micro titer plates were used and a serial two-fold dilution of 25 µL of serum in equal volumes of TBS was prepared. After adding 25 µL of the 1.5% erythrocyte suspension the plates were incubated at room temperature (30±2 °C) for 1 h 30 min. The highest dilution of the sample showing agglutination with the erythrocytes was recorded as the hemagglutination titer, which was the reciprocal of the dilution. Titers were calculated as the median of triplicate values across five independent determinations.

Optimal conditions for hemagglutination activity of serum

To investigate the impact of cations on hemagglutination activity, the concentrations of different divalent cations were examined, TBS I (50 mM Tris, 100 mM NaCl, 5 mM MgCl₂), TBS II

(50 mM Tris, 100 mM NaCl, 5 mM BaCl₂), TBS III (50 mM Tris, 100 mM NaCl, 1 mM MnCl₂), TBS IV (50 mM Tris, 100 mM NaCl, 10 mM KCl) and TBS V (50 mM Tris, 100 mM NaCl, 10 mM CaCl₂). Various concentrations of CaCl₂ ranging from 1 to 12.5 mM were tested in TBS. Additionally, the effect of different pH levels (ranging from 5.0 to 9.0) of TBS-V (50 mM Tris, 100 mM NaCl, 10 mM CaCl₂, pH 7.5) was evaluated. In order to investigate the impact of temperature on hemagglutination activity a serum sample were incubated at temperatures ranging from 10 to 60 °C.

Hemagglutination inhibition (HAI) assay

The highest reciprocal of the lowest dilution of inhibitors giving complete inhibition expressed the HAI assay. Test sugars (200 mM) or glycoproteins (10 mg.mL⁻¹) were serially diluted in equal volumes of serum sample in TBS-V for 1 hour, followed by the addition of 25 µL of 1.5% buffalo erythrocytes diluted to sub-agglutinating concentration (four titre values) for 1 h 30 min at room temperature (30±2 °C).

Lysozyme activity

The lysozyme activity of the serum was assessed by following the method described by Hultmark *et al*⁵¹ utilizing a bacterial suspension of *Micrococcus lysodeikticus* (ATCC No. 4698; Sigma). Different concentrations of serum (5 to 25 µl) were prepared separately and combined with bacterial suspension of *M. lysodeikticus* (0.2 mg.mL⁻¹) in 50 mM phosphate buffer pH 6.5 to reach a final volume of 200 µl. A control without serum was incubated under the same conditions. The reaction mixtures were incubated for varying time intervals ranging from 5 to 60 minutes at room temperature (30±2 °C). Absorbance was recorded at 450 nm using a BIO-RAD ELISA micro plate reader to determine the time-dependent decline in turbidity due to bacterial cell lysis.

Antimicrobial activity

Microbial strains media and culture conditions

Bacterial isolates were collected from crabs exhibiting visible cuticular whereas hemolymph serum for antimicrobial assays was obtained exclusively from healthy, uninjured intermoult crabs. Bacterial isolates obtained from the infected or injured cuticular region of freshwater crab *B. cunicularis* was carefully excised, homogenized using a sterile mortar and pestle in 0.9% saline, and centrifuged at 1800 rpm for 10 min. The resulting

supernatant was streaked onto selective agar plates, including Thiosulphate Citrate Bile Salt (TCBS) agar, Eosin Methylene Blue (EMB) agar and Cetrimide agar for identification based on their morphology, size and pigmentation following the methods described by Holt *et al.*⁵² Additionally, three fungal strains, namely *Candida albicans* (KT831886), *C. tropicalis* (OK174311) and *A. fumigatus* were suspended in Mueller Hinton broth and cultured at 37 °C for 24 h. The fungal cultures were maintained under these conditions for preliminary antifungal screening and to ensure uniformity with bacterial antimicrobial assays. It is acknowledged that these conditions do not represent optimal growth requirements for filamentous fungi.

Antimicrobial assays

The agar well diffusion method was performed on each culture, following⁵³. Muller Hinton agar (MHA) plates were prepared and sterile swabs were used to spread the (24 hours) old broth cultures of bacteria and fungi onto the plates. Bacterial and yeast cultures were standardized to 0.5 McFarland turbidity prior to inoculation. Mueller Hinton agar was employed for antifungal assays to maintain methodological consistency with bacterial assays and to facilitate comparative analysis of antimicrobial activity. Wells were created on the agar using a cork borer. To assess the antibacterial activity of the serum the experimental setup was as follows, Streptomycin ($\text{mg}\cdot\text{mL}^{-1}$) and Amphotericin B ($\text{mg}\cdot\text{mL}^{-1}$) were used as positive controls, while Tris-HCl buffer (10 mM, pH 7.5) served as the negative control. In brief, 100 μL of serum sample from *B. cunicularis* at different concentrations (45, 65 and 85 $\text{mg}\cdot\text{mL}^{-1}$) were loaded into the wells of MHA medium. The plates were subsequently placed in an incubator at 37 °C for 24 hours. The antibacterial activity was assessed by measuring the diameter of the zone of inhibition using a standard zone scale in millimetres (mm).

Antimicrobial agglutination assay

The antimicrobial agglutinating activity was determined in U-bottom microtiter plate (Griener, Nürtingen, Germany) by serial two-fold dilution. Briefly, 50 μL of serum was mixed with an equal volume of TBS-V (50 mM Tris-HCl; 100 mM NaCl; 10 mM CaCl_2 ; pH 7.5). After dilution, 50 μL of bacterial and fungal suspension (0.5 O.D. at 570 nm) were added to each well, incubated for 1 h at room temperature (30 ± 2 °C) and kept overnight at 10 °C,

then the Agglutination was observed under light microscope (Labomed microscope).

Statistical analysis

All data were expressed as mean \pm SD of triplicate experiments from five determinations using (GraphPad Prism 8.4.9). Statistical significance was determined using one-way ANOVA P -value < 0.05 was considered statistically significant.

Results

Biochemical constituents

Biochemical analysis of the serum from *Barytelphusa cunicularis* revealed that proteins were the most abundant macromolecules, with a concentration of 92.80 $\text{mg}\cdot\text{mL}^{-1}$, followed by carbohydrates at 38.81 $\text{mg}\cdot\text{mL}^{-1}$ and lipids at a minimal concentration of 2.25 $\text{mg}\cdot\text{mL}^{-1}$ (Fig. 1).

PO activity

Serum PO activity with various substrates

The serum obtained from the hemolymph of *B. cunicularis* exhibited the highest activity at 455 nm when exposed to DL-DOPA, compared to the activity observed with L-DOPA (455 nm), dopamine (400 nm) and catechol (455 nm) of diphenols. In contrast, the serum showed minimal oxidation towards monophenols like tyramine (405 nm) and L-tyrosine (420 nm) as well as polyphenols such as hydroquinone (410 nm) and pyrogallol (400 nm). As DL-DOPA demonstrated higher oxidative activity, it was chosen as the substrate to assess the presence of PO activity in all subsequent experiments (Fig. 2).

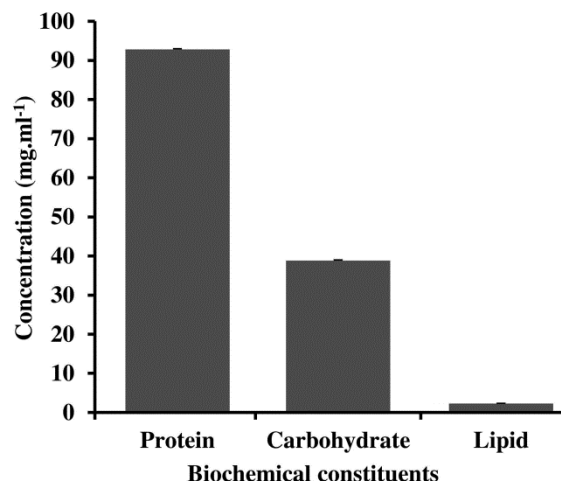


Fig. 1 — Graphical representation of biochemical compositions of serum from the freshwater crab *B. cunicularis*.

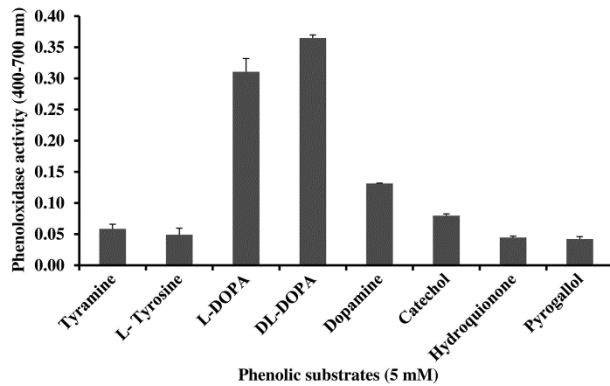


Fig. 2 — Phenoloxidase activity of the serum from the *B. Cucicularison* different phenolic substrates (5 mM) in Tris-HCl buffer (Tris 50 mM, pH 7.5) incubated at 25 °C for 10 min and absorbance at 400–700 nm.

Effect on optimal conditions for PO measurement of serum

The serum PO activity of *B. cucicularis* exhibited an increase when exposed to concentrations ranging from 1.0 to 10 mM of DL-DOPA. Among the tested concentrations 7.5 mM was determined as the optimal concentration and was subsequently utilized for further testing (Fig. 3A). Additionally, the PO activity was assessed at various time intervals (1-45 min) using DL-DOPA. The highest PO activity was observed at the 10 min indicating it to be the optimal incubation time (Fig. 3B). Furthermore, the PO activity was evaluated using Tris-HCl buffer with varying ionic strengths (10-60 mM). The highest activity was recorded at 50 mM thus establishing it as the optimal buffer concentration for subsequent experiments (Fig. 3C). Similarly, PO activity was assessed using DL-DOPA at different pH values ranging from 6.0 to 9.0 within Tris-HCl buffer (50 mM). The highest PO activity was observed at pH 7.5 (Fig. 3D). To determine the effect of temperature on serum incubation was conducted at various temperatures from 10 to 80 °C. Stable and peak PO activity was observed at 25 °C, signifying this temperature as the optimal condition (Fig. 3E). In summary, the optimal conditions for measuring serum PO activity on DL-DOPA (7.5 mM) were as follows, 50 mM Tris-HCl buffer, pH 7.5, incubation at 25 °C for 10 min, with absorbance readings taken at 455 nm.

Effect of inhibition in PO activity

A significant reduction in serum PO activity was observed at 5 mM PTU compared to the control and other concentrations tested ($P < 0.05$) (Fig. 4).

Effect of activation on PO activity

Serum phenoloxidase (PO) activity was stimulated by various concentrations of exogenous proteases and

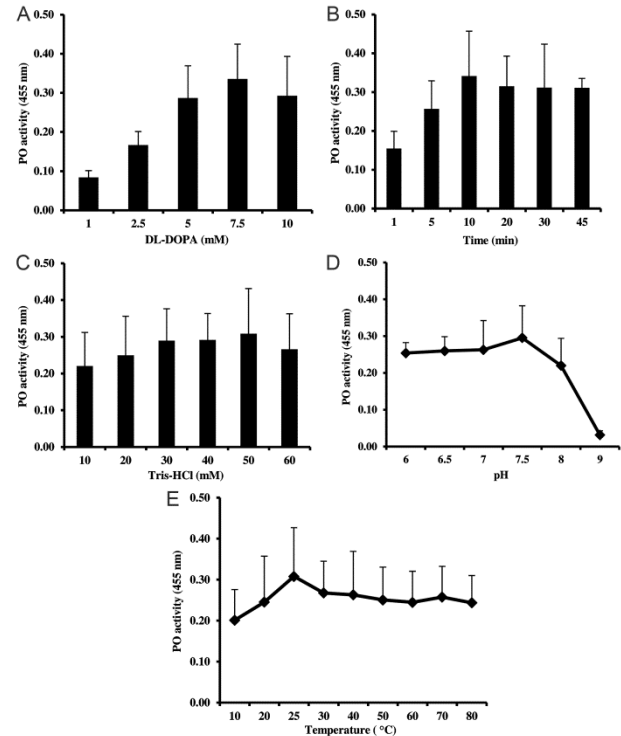


Fig. 3 — Effect on optimal conditions on oxidation of DL-DOPA (A) different concentrations of DL-DOPA, (B) time interval, (C) ionic strength, (D) different pH, (E) temperature of PO activity in the serum of *B. cucicularis*. Data represents mean of triplicate repeats of five determinations (mean \pm SD).

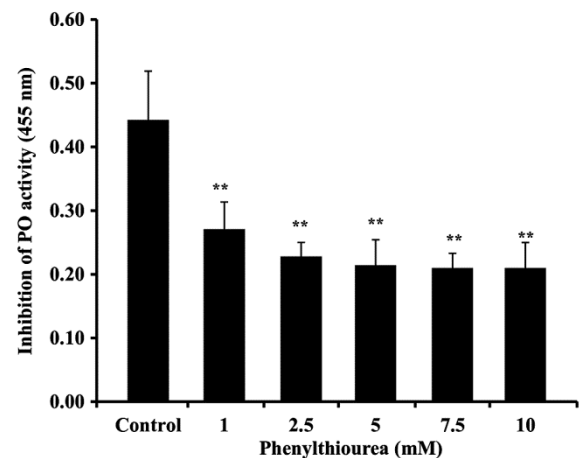


Fig. 4 — Inhibition of phenoloxidase activity using PTU in the serum of *B. cucicularis*. Data represents mean of triplicate repeats of five determinations (mean \pm SD). Statistical significance was determined using one-way ANOVA followed by appropriate post-hoc analysis. ** $P < 0.01$ was considered statistically significant.

immune stimulants. A significant increase in PO activity was observed with 10 mg of trypsin (Fig. 5A), 5 mg of the anionic detergent SDS (Fig. 5B), and

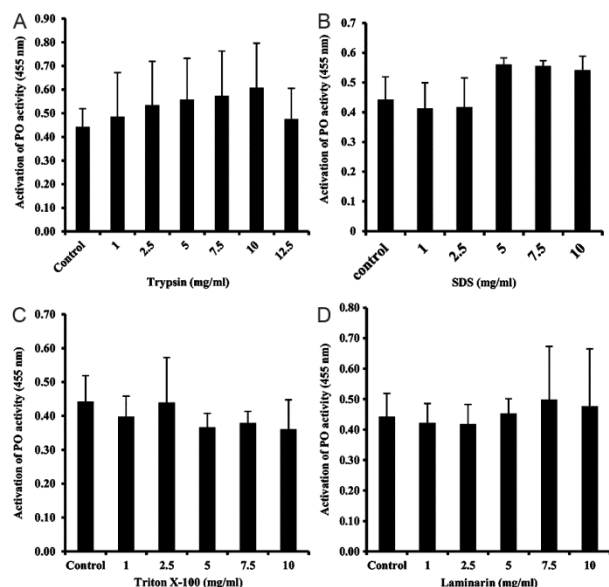


Fig. 5 — Activation of phenoloxidase activity (A) trypsin, (B) SDS, (C) Triton X-100, (D) laminarin in the serum of the freshwater crab *B. cunicularis*. Data represents mean of triplicate repeats of five determinations (mean \pm SD). Statistical significance was determined using one-way ANOVA followed by appropriate post-hoc analysis. * $P < 0.05$, ** $P < 0.01$ was considered statistically significant.

Table 1 — Hemagglutinating activity in the serum from the hemolymph of freshwater crab *B. Cunicularis* against various mammalian erythrocyte types tested

Hemagglutination (HA) activity	
Erythrocyte types tested	Titer value*
Human A	16
Human B	64
Human O	32
Chicken	16
Goat	-
Sheep	16
Cow	64
Ox	32
Buffalo	512

Table 2 — Effect of cations on hemagglutination activity in the serum of *B. cunicularis*

Erythrocyte types tested	Hemagglutination (HA) activity				
	Tris 50 mM, NaCl 100 mM, pH 7.5 with				
	5 mM MgCl ₂	5 Mm BaCl ₂	1 mM MnCl ₂	10 mM KCl	10 mM CaCl ₂
Human A	128	2	4	64	32
Human B	4	2	4	8	128
Human O	16	2	4	0	64
Chicken	2	32	32	8	32
Goat	-	-	-	-	-
Sheep	2	-	2	4	32
Cow	2	2	4	4	128
Ox	2	2	8	8	64
Buffalo	32	8	16	32	1024

7.5 mg of the non-self molecule laminarin (Fig. 5C), compared to the control. In contrast, the cationic detergent Triton X-100 did not induce any activation of PO activity (Fig. 5D).

Hemagglutination assay

HA activity

The HA activity was observed when the serum (25 μ l) was serially diluted in TBS (Tris 50 mM, NaCl 100 mM, pH 7.5) along with various mammalian erythrocyte suspensions. The HA activity of the crab serum with different mammalian erythrocytes is presented in (Table 1). The maximum agglutination activity was observed with Buffalo erythrocytes reaching a titre value of 512.

Effect of cations on hemagglutination activity

The hemagglutination activity of the serum (25 μ l) was evaluated through serial dilutions in TBS (Tris 50 mM, NaCl 100 mM, pH 7.5) containing various concentrations of different cations, including TBS-I to TBS-V MgCl₂, BaCl₂, MnCl₂, KCl and CaCl₂, using erythrocyte suspension. Among the tested metal ions as shown in (Table 2), the maximum hemagglutination activity was observed with a titer value of 1024 in buffalo erythrocyte when using 10 mM CaCl₂ with TBS, surpassing the other metal ions. Enhanced titers observed in the presence of specific cations indicate cation enhanced rather than strictly cation dependent lectin activity.

Effect on optimal conditions for hemagglutination activity of serum

The serum of *B. cunicularis* displayed an increase in hemagglutination activity when exposed to concentrations ranging from 1.0 to 12.5 mM of CaCl₂. Among the tested concentrations, 10 mM was identified as the optimal concentration and was subsequently used for further testing (Table 3).

Erythrocyte types tested	Hemagglutination (HA) activity titer value					
	1 mM CaCl ₂	2.5 mM CaCl ₂	5 mM CaCl ₂	7.5 Mm CaCl ₂	10 mM CaCl ₂	12.5 mM CaCl ₂
Human A	4	8	16	16	32	16
Human B	4	8	32	64	128	64
Human O	16	16	32	32	64	16
Chicken	-	2	4	16	32	16
Goat	-	-	-	-	-	-
Sheep	2	8	16	16	32	16
Cow	16	32	64	64	128	64
Ox	2	4	4	4	64	32
Buffalo	128	256	512	512	1024	256

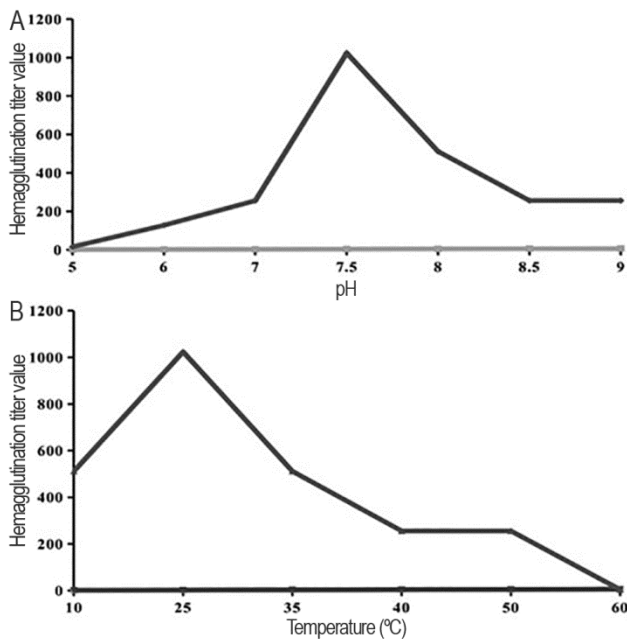


Fig. 6 — Effect on optimal conditions of hemagglutinating activity (A) different pH, (B) different temperature in the serum of *B. cunicularis*. Data represents average titre value of five determinations.

Consequently, Buffalo erythrocytes were selected as the indicator cells for further investigation. To assess the different pH of HA activity in the serum was serially diluted in TBS-V (Tris 50 mM, NaCl 100 mM, CaCl₂ 10 mM) adjusted to pH levels ranging from 5.0 to 9.0. The highest hemagglutination activity was observed at pH 7.5 with a buffalo erythrocyte titer value of 1024 (Fig. 6A). To, investigate the impact of temperature on hemagglutination activity incubation was performed at various temperatures ranging from 10 to 60 °C. The most stable and highest titer value of 1024 with Buffalo erythrocytes was observed at 25 °C indicating that this temperature represents the optimal condition (Fig. 6B).

Hemagglutination inhibition (HAI) assay

Among the 10 simple sugars tested, it was found that all of them, except for D-mannose inhibited agglutination. The highest activity was observed with D-galactose at a concentration of 6.25 mM in the serum against buffalo erythrocytes. In the case of 3 amino sugars and 3 *N*-acetyl derivatives of sugars, D-galactosamine and *N*-acetyl-D-galactosamine the highest inhibitory agglutinating activity against the serum was observed at concentrations of 6.25 mM. When testing glycoproteins namely Fetuin, Thyroglobulin and BSM show no hemagglutination inhibition (HAI) activity against serum (Table 4).

Lysozyme activity

In this study, we assessed the lysozyme activity of serum derived from *B. cunicularis* by measuring its impact on a bacterial suspension of *Micrococcus lysodeikticus*. The absorbance indicative of lysozyme activity was monitored over different time intervals using varying concentrations of serum (5 to 25 µl). Our results revealed a gradual decrease in lysozyme activity across the range of 5 to 25 µl of serum, with the highest reduction observed at 60 minutes with 25 µl of serum (Fig. 7).

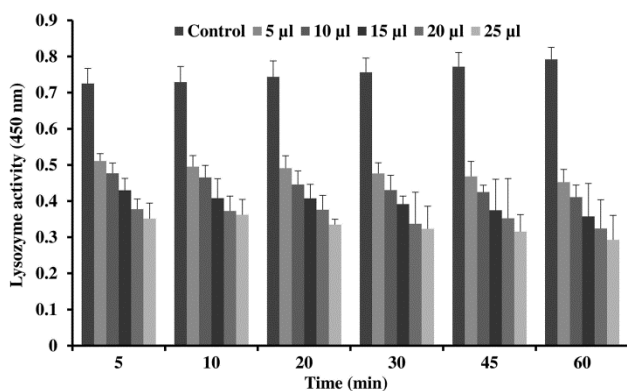
Antibacterial activity

Bacterial colonies isolated from wounded sites of crab

Scrap tissue samples were collected from the Infected or wounded cuticle sites of freshwater crab *B. cunicularis* and cultured on three different selective media: TCBS-agar, EMB-agar and Cetrimide-agar medium. These media supported the growth of bacterial colonies, which displayed variations in size (small or large) and pigmentation (yellow, green, metallic green, or greenish yellow). In TCBS-agar, three distinct types of colonies were observed: *Vibrio* sp. 1 dark green (large) (Fig. 8A), *Vibrio* sp. 2 light

Table 4 — Hemagglutination inhibition activity of the serum of *B. Cunicularis* (Hemagglutination titer =4) by carbohydrates and glycoproteins against buffalo RBC tested

Carbohydrates and glycoproteins tested	Maximum concentration tested	Minimum inhibitory concentration
Simple sugars (mM)		
D- glucose	200	50
D-galactose	200	6.25
D-mannose	200	-
D- fructose	200	100
D-xylose	200	50
D-arabinose	200	100
D-raffinose	200	12.5
D- cellobiose	200	25
Lactose	200	12.5
Sucrose	200	25
Amino Sugars (mM)		
D- glucosamine	200	-
D- galactosamine	200	6.25
D- mannosamine	200	-
N- acetyl amino sugars (mM)		
N-acetyl-D-glucosamine	200	-
N- acetyl-D-galactosamine	200	6.25
N- acetyl-D-mannosamine	200	-
Glycoproteins (10 mg.ml ⁻¹)		
Bovine sub maxillary mucin	10	-
Thyroglobulin	10	-
Fetuin	10	-

Fig. 7 — Lysozyme activity of the serum from the freshwater crab *B. cunicularis* against *Micrococcus lysodeikticus*. Data represents mean of triplicate repeats of five determinations (mean \pm SD) using serum against bacterial suspension alone in control.

green (small) (Fig. 8B) and *Vibrio* sp. 3 yellow (large) (Fig. 8C) colonies under a light microscope, these colonies were identified as three types of *Vibrio* spp., The colonies that grew well on EMB-agar were identified as *Escherichia coli* based on their growth characteristics and the metallic green coloration of the colonies in Methylene blue under strong acidic conditions, which is indicative of lactose fermenters (Fig. 8D). The colonies that grew on Cetrimide-agar

with green pigmentation were identified as *P. aeruginosa* (Fig. 8E). In summary, all five types of colonies with distinct colours and morphologies were observed at wounded cuticle sites of freshwater crab *B. cunicularis*.

Antimicrobial Activity

Antimicrobial activity of the serum obtained from *B. cunicularis* was assessed by an agar well diffusion method. Investigations were performed against a range of five different bacteria: *Vibrio* sp., 1, 2, 3, *E. coli* and *P. aeruginosa* and clinical pathogens of three fungal strains *C. albicans*, *C. tropicalis* and *A. fumigatus*. The results obtained from the experiments conducted at different concentration of serum such as (45, 65 and 85 mg.mL⁻¹) were evaluated and compared to the positive controls such as streptomycin and Amphotericin B are shown in (Fig. 9A-H) and (Table 5). Antimicrobial activity was shown against five bacterial strains and two fungal pathogens compared to all *E. coli* and *P. aeruginosa* shows highest activity of 18 mm in *E. coli* (85 mg.mL⁻¹) while no activity was observed against *C. albicans*.

Microbial agglutination activity

Serum agglutinated with all the five bacterial species isolated from *B. cunicularis* such as *Vibrio* sp.

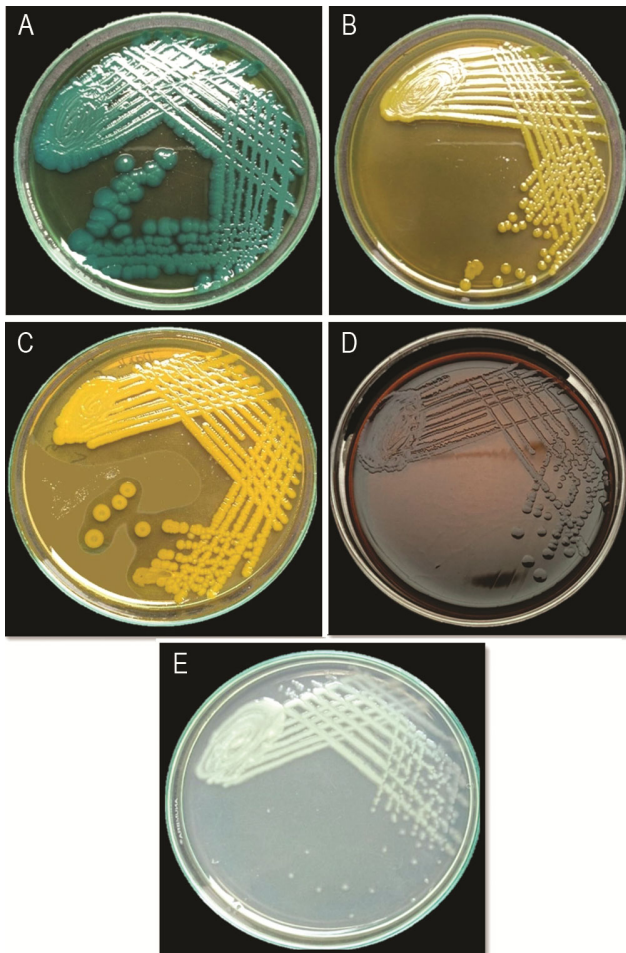


Fig. 8 — Bacterial colonies isolated from injured/wounded sites of freshwater crab of *B. cunicularis* Culture grown in selective media of (A) *Vibrio* sp. 1 (TCBS agar), (B) *Vibrio* sp. 2(TCBS agar), (C)*Vibrio* sp. 3(TCBS agar), (D) *E. Coli* (EMB agar), (E) *P. aeruginosa* (Cetrinimide agar).

1, 2, 3, *E. coli* and *P. aeruginosa* with titres of 512, 256, 64,512 and 32. Serum also agglutinated fungi *C. albicans*, *C. tropicalis* and *A. fumigatus* with titres of 64, 26 and 16respectively (Table 6). The results clearly show that the highest agglutination activities were recorded with *Vibrio* sp. 1and *E. coli* (titer: 512)

Discussion

The freshwater crab *Barytelphusa cunicularis* is traditionally valued for both its nutritional and medicinal significance, being rich in proteins for the poor crabs are rich in essential macronutrients and micronutrients, particularly protein, which plays crucial roles in tissue repair, muscle maintenance and immune modulation⁵. In this study, serum analysis revealed protein levels significantly higher than carbohydrate and lipid concentrations, consistent with

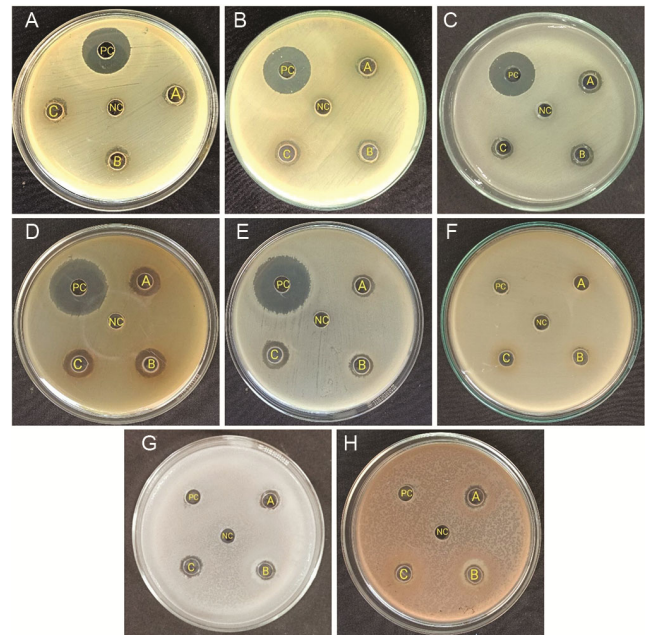


Fig. 9 — Antimicrobial activity of serum from *B. cunicularis* showing zone of inhibition (mm) against (A) *Vibrio* sp. 1, (B) *Vibrio* sp. 2, (C) *Vibrio* sp. 3, (D) *E. Coli*, (E) *P. aeruginosa*, (F) *C. albicans*, (G) *C. tropicalis*, (H) *A. fumigatus*. Data represents mean \pm SD of three determinations using serum sample from different preparations in the zone of inhibition (mm).

reports on the hemolymph composition of *Dromia dehaani*, and serum constituents in *Oziotelphusa senex* and *Scylla paramamosain*⁵⁵. Hemocytes are central to crustacean innate immunity, mediating phagocytosis, encapsulation and nodule formation, and serving as the primary source of prophenoloxidase and other humoral effectors, which together help *B. cunicularis* defend against bacterial and fungal pathogens⁵⁵. The freshwater and marine crustacean immune functions of serum, plasma, hemocyanin and lectin has been reported^{9,10,56,57}. Phenoloxidase in crustaceans comes under type 3 copper proteins and they are classified into two types, one from hemocytes released as proPO¹⁵ and other derived from plasma and hemocyanin^{9,17}. The serum PO activity of *B. cunicularis* was higher towards the diphenol DL-DOPA at 455nm indicating catechol oxidase type of PO respectively. Likewise, the serum of *Albunea symmysta* and hemocyte lysate supernatant of *Macrobrachium rosenbergii* exhibited higher affinity towards DL-DOPA at 490nm^{21,14}. However, in recent reports of serum, plasma and hemocyanin obtained from the hemolymph of most crustaceans displayed greater affinity towards the diphenol L-DOPA in the range of 400-500 nm

respectively^{17,13}. Furthermore, the optimal substrate concentration of DL-DOPA was estimated at 5mM similar to *M. rosenbergii*¹⁷ and *A. symmysta*²¹. For PO activity to happen in crustacean's pH, temperature, time of incubation, ionic strength and buffer system plays a crucial role that can either enhance or decline the PO activity⁵⁸. So it is essential to optimize the parameters of Phenoloxidase (PO) activity for each specific crustacean species. In the present study, serum of *B. cunicularis* gave maximum activity in pH 7.5 similar to *M. rosenbergii* and *A. symmysta*^{17,21}.

Similarly, the optimum temperature was found to be 25°C consistent to the PO activity of lectin in *Paratelphusa jacquemontii*¹⁵ and *Metapenaeus dobsoni*⁵⁷. As a significant parameter, the time essential for favourable PO activity was found to be 10 min consistent with *M. dobsoni* for 15 min respectively⁵⁷. The ionic strength of 50 mM Tris-HCl influenced higher activity differing from the previous report in *Scylla olivacea* at 10 mM⁵⁹, respectively. Interestingly, comparative inhibition of serum phenoloxidase by sulphur-containing compound, 1-phenyl-2-thiourea (PTU) indicates the sensitivity of the enzyme towards PTU and confirms the copper oxidase activity. Together, the use of specific substrates and modulators enabled functional characterization of phenoloxidase activity and its physiological activation mechanisms in *B. cunicularis* as copper is present in its active site^{14,20,13}. On the other hand, effect of activators such as trypsin (protease) and SDS (anionic detergent) enhanced the activity of PO similar to *P. jacquemontii*¹⁵ and *M. rosenbergii*^{13,14}. However, laminarin from *Laminaria digitata* and a non-ionic detergent like Triton X-100 failed to produce significant activation contrast to *A. symmysta*²¹. These results indicate that trypsin induces higher PO activity by proteolytic cleavage or by conformational changes.

Besides PO system, the agglutination reaction exhibited by the serum confirms the presence of agglutinin molecule such as lectins⁶⁰ or plasma and hemocyanin^{10,61}. The present study on *Barytelphusa cunicularis*, showed a markedly higher hemagglutination titer (1024) with buffalo erythrocytes at 10 mM CaCl₂ and pH 7.5 compared to the earlier report by Priyadharshini (2018), which recorded a lower titer (256) with rabbit erythrocytes. The increased activity observed here may be due to serum-based analysis and optimized assay conditions. Unlike the previously reported sialic acid-specific

agglutinin, the present study indicates galactose-binding specificity. These results suggest functional heterogeneity or modulation of lectin activity within the same species under varying physiological and experimental conditions⁶². The serum displayed highest titre value against buffalo erythrocytes consistent with previous observations in *Portunus reticulatus*⁶³, *Atergatis integerrimus*⁶⁴ and plasma or hemocyanin *M. rosenbergii*^{18,10}. However, agglutinins from marine and freshwater crustaceans reported to possess more affinity towards rabbit erythrocytes^{65,66,67} indicating the diversity and uniqueness of each agglutinin. The agglutinin requirement of Ca²⁺ for its activity depicts the presence of calcium dependent lectin or C-type lectin which is widely present in crustaceans^{62,68}. The use of erythrocytes from multiple species was intended to assess the carbohydrate-binding specificity and functional diversity of crustacean lectins, which are known to act as important pattern recognition receptors in innate immunity, binding diverse glycan structures on cell surfaces and participating in pathogen recognition and hemagglutination^{69,70}. Sugar binding specificity of agglutinin detected by inhibition assay using buffalo erythrocytes revealed the highest inhibition for D-galactose, D-galactosamine and N-acetyl-D-galactosamine at 6.25 mM concentration indicating it higher affinity for galactose sugars. Unlike other crustacean agglutinin the serum failed to show maximum inhibition towards glycoproteins such as bovine sub maxillary mucin^{62,71} and fetuin^{68,10}.

Serum lysozyme activity peaked at 60 minutes with 25 µL, showing effective lysis of *Micrococcus lysodeikticus*. Similar lysozyme-like activity has been reported in C-type lysozyme from *Fenneropenaeus penicillatus* and *Scylla paramamosain*^{72,73}. HLS from *P. jacquemontii*¹⁵, support the current findings. Arthropods represent a significant and largely unexplored source of antimicrobial peptides and molecular mechanisms of action⁷⁴. In this study, five bacterial strains from the wounded cuticle of the freshwater crab *B. cunicularis* were isolated and cultured. Subsequently, antibacterial testing revealed potent activity of *B. cunicularis* serum against gram-negative bacteria, especially *E. coli* (17-18mm), which aligns with observations from *Paratelphusa hydrodromous*⁷⁵. While *C. albicans* showed no inhibition, other fungal strains like *A. fumigatus* responded, although antifungal activity should be

interpreted cautiously due to non-optimal incubation conditions, paralleling the action of lectins (Pp-Lec) and peptides (Fc-hsl) from *P. pelagicus*⁷⁶, Marine crabs like *Dromia dehaani* also demonstrate antifungal properties⁷⁷.

Agglutination of bacterial and fungal strains by the serum of *B. cunicularis* further supports its immune potential. The highest agglutination titres were observed for *Vibrio* sp. 1 and *E. coli*, indicating a strong affinity toward lipopolysaccharide (LPS) structures present on gram-negative bacterial membranes. This suggests that the agglutinating component in the serum likely binds to microbial surface molecules, particularly LPS. Similar mechanisms have been reported in crustaceans; for instance, lectin (Md-Lec) from *Metapenaeus dobsoni* was shown to bind LPS and agglutinate gram-negative strains such as *V. parahaemolyticus* and *A. hydrophila*⁵⁷. Likewise, lectin from *Penaeus semisulcatus* agglutinated both gram-negative and gram-positive bacteria, possibly through interaction with LPS or structurally similar moieties such as sialic acid-Neu5A⁶³. C-type lectins from *Procambarus clarkii* also agglutinated *S. aureus* and *V. alginolyticus* in a calcium-dependent manner⁷⁸. Furthermore, hemocyanin from *Marsupenaeus japonicus* exhibited broad agglutination activity against both gram-positive and gram-negative bacteria¹⁹.

Conclusion

The hemolymph serum of *Barytelphusa cunicularis* exhibits a multifaceted innate immune system, including phenoloxidase, lectins, lysozyme and antimicrobial compounds. These components act synergistically to recognize, agglutinate and neutralize a broad range of microbial pathogens. Optimization of assay conditions for PO activity and hemagglutination confirms the functional robustness of the serum. Overall, these findings demonstrate the immune competence of *B. cunicularis* and provide a foundation for future studies on immune effectors, with potential applications in aquaculture health management and antimicrobial defense.

Acknowledgement

We thank the local people of Muthanur Village, Yelagiri, for their assistance in collecting crabs. We are also grateful to the Zoological Survey of India (ZSI), Chennai, for their support in the taxonomic identification of the freshwater crab *Barytelphusa cunicularis*.

Credit authorship contribution statement

SS Execution, writing original draft of the manuscript and analysis of all the experimental works. RS Guidance, conception, design, examining the experimental works, visualization and laboratory providing. MRS Reviewing, investigation. FAA Editing, software. All authors read and approved the final manuscript.

Funding

Not available

Availability of data and material

All data generated or analysed during this study are included in this published article

Ethical approval

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

- 1 Food and Agriculture Organization of the United Nations (FAO), *The state of world fisheries and aquaculture: Towards blue transformation*. FAO, (2022) 1.
- 2 Boyd CE, McNeven AA & Davis RP. The contribution of fisheries and aquaculture to the global protein supply. *Food Secur*, 14 (2022) 805.
- 3 Sharma A, Thakur K, Sharma AK, Singh B & Kumar R. Role of crustaceans in traditional medicine and extensive applications of their byproducts: A comprehensive review. *Life Res*, 8 (2025) 16.
- 4 Verdegem M, Buschmann AH, Latt UW, Dalsgaard AJ & Lovatelli A. The contribution of aquaculture systems to global aquaculture production. *J World Aquacult Soc*, 54 (2023) 206.
- 5 Chavan SV. Study of natural products from freshwater biodiversity as a recent strategy: An overview. *World J Pharm Res*, 11 (2022) 395.
- 6 Nanda PK, Das AK, Dandapat P, Dhar P, Bandyopadhyay S, Dib AL, Lorenzo JM & Gagaoua M. Nutritional aspects, flavour profile and health benefits of crab meat based novel food products and valorisation of processing waste to wealth: A review. *Trends Food Sci Technol*, 112 (2021) 252.
- 7 Shibana C, Subavathy P & Thilaga RD. Biochemical composition of the marine crab *Portunus pelagicus* from Gulf of Mannar. *Int J Sci Res*, 7 (2018) 318.
- 8 Aaqillah-Amr MA, Hidir A, Noordiyana MN & Ikhwanuddin M. Morphological, biochemical and

- histological analysis of mud crab ovary and hepatopancreas at different stages of development. *Anim Reprod Sci*, 195 (2018) 274.
- 9 Sivakumar MR, Denis M, Shanthi S & Munusamy A. Phenoloxidase activity in humoral plasma, hemocyanin and hemocyanin separated proteins of the giant freshwater prawn *Macrobrachium rosenbergii*. *Int J Biol Macromol*, 102 (2017) 977–985.
 - 10 Sivakumar MR, Denis M, Shanthi S & Arumugam M. Agglutination of plasma, hemocyanin and separated hemocyanin from the hemolymph of the freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879). *J Crustac Biol*, 40 (2020) 309.
 - 11 Huang Z, Aweya JJ, Zhu C, Tran NT, Hong Y, Li S, Yao D & Zhang Y. Modulation of crustacean innate immune response by amino acids and their metabolites: inferences from other species. *Front Immunol*, 5 (2020) 574721.
 - 12 Kulkarni A, Krishnan S, Anand D, Kokkattunivarthil Uthaman S, Otta SK, Karunasagar I & Kooloth Valappil R. Immune responses and immune protection in crustaceans with special reference to shrimp. *Rev Aquacult*, 13 (2021) 431.
 - 13 Sivakumar MR & Shanthi R. Plasma and Hemocyanin Phenoloxidase Derived from the Hemolymph of Giant Freshwater Prawn *Macrobrachium rosenbergii* (De Man, 1879). In *Pertinent and Traditional Approaches Towards Fishery* (IntechOpen, London), (2022)1
 - 14 Sivakumar MR, Abisha Adline F & Shanthi R. Hemocytes lysate supernatant derived phenoloxidase activity in the hemolymph of giant freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879). *Int J Zool Anim Biol*, 5 (2022) 000403.
 - 15 Denis M, Sivakumar MR, Tamilarasan K, Karthigayani T & Prabu M. Activation of phenoloxidase activity by humoral lectin in hemocytes of freshwater crab *Paratelphusa jacquemontii*. *Int J Biol Macromol*, 97 (2017) 258.
 - 16 Panigrahi A, Das RR, Sundaram M, Sivakumar MR, Jannathulla R, Lalramchhani C, Antony J, Anand PS, Kumar KV, Jayanthi M & Dayal JS. Cellular and molecular immune response and production performance of Indian white shrimp *Penaeus indicus* reared in a biofloc-based system with different protein levels of feed. *Fish Shellfish Immunol*, 119 (2021) 31.
 - 17 Sivakumar MR & Shanthi R. Serum phenoloxidase activity in the hemolymph of giant freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879). *Int J Zool Anim Biol*, 5 (2022) 000419.
 - 18 Mohanty J, Sahoo S, Badhe MR, Pillai BR, Sahoo PK, Suryawanshi AR & Patnaik BB. Lectin-like activity of hemocyanin in freshwater prawn *Macrobrachium rosenbergii*. *Protein J*, 39 (2020) 358.
 - 19 Zhao X, Qiao J, Zhang P, Zhang Z, Aweya JJ, Chen X, Zhao Y & Zhang Y. Protein diversity and immune specificity of hemocyanin from shrimp *Litopenaeus vannamei*. *Front Immunol*, 12 (2021) 772091.
 - 20 Shanthi S, Sivakumar MR & Rayvathy B. Serum phenoloxidase activity in the hemolymph of the anomuran crab *Albunea symmysta* (Linnaeus, 1758). *J Crustac Biol*, 41 (2021) ruab003.
 - 21 Adline FA, Shanthi R, Sivakumar MR, Roshni K & Sowmiya S. *In vitro* immune analysis of serum from the hemolymph of the anomuran crab *Albunea symmysta* (Linnaeus, 1758) displayed diversified reactions. *J Invertebr Pathol*, 204 (2024) 108098.
 - 22 Sanchez-Salgado JL, Pereyra MA, Agundis C, Calzada-Ruiz M, Kantun-Briceno E & Zenteno E. *In vivo* administration of LPS and β -glucan generates the expression of a serum lectin and its cellular receptor in *Cherax quadricarinatus*. *Fish Shellfish Immunol*, 94 (2019) 10.
 - 23 Durairaj KR, Saravanan K, Mohan K & Ravichandran S. Purification, characterization and biological functions of metalloprotein isolated from haemolymph of mud crab *Scylla serrata* (Forsk., 1775). *Int J Biol Macromol*, 164 (2020) 3901.
 - 24 Tran NT, Liang H, Li J, Deng T, Bakky MAH, Zhang M & Li S. Cellular responses in crustaceans under white spot syndrome virus infection. *Fish Shellfish Immunol*, 140 (2023) 108984.
 - 25 Ratcliffe NA. The prophenoloxidase system and its role in arthropod immunity. *Phylogenes Immune Funct*, (2024) 45.
 - 26 KV R, K S, Deepika A & Kulkarni A. Shrimp immune system and immune responses. *Fish Immune Syst Vaccines*, (2022) 17.
 - 27 Bostjancic LL, Francesconi C, Rutz C, Hoffbeck L, Poidevin L, Kress A & Theissinger K. Host-pathogen coevolution drives innate immune response to *Aphanomyces astaci* infection in freshwater crayfish. *BMC Genomics*, 23 (2022) 600.
 - 28 Ahmmed MK, Bhowmik S, Giteru SG, Zilani MNH, Adadi P, Islam SS, Kanwugu ON, Haq M, Ahmmed F, Ng CCW, Chan YS, Asadujjaman M, Chan GHH, Naude R, Bekhit AEA, Ng TB & Wong JH. An update of lectins from marine organisms: characterization, extraction methodology and potential biofunctional applications. *Mar Drugs*, 20 (2022) 430.
 - 29 Guzman-Tellez P, Martinez-Castillo M, Flores-Huerta N, Rosales-Morgan G, Pacheco-Yepez J, La Garza M, Serrano-Luna J & Shibayama M. Lectins as virulence factors in *Entamoeba histolytica* and free-living amoebae. *Future Microbiol*, 15 (2020) 919.
 - 30 Chen HY, Li WY, Wang J, Bo GW, Yang GW & Yang HT. A C-type lectin containing two carbohydrate recognition domains participates in antibacterial response by regulating JNK pathway and promoting phagocytosis. *Fish Shellfish Immunol*, 127 (2022) 349.
 - 31 Singrang N, Laophetsakunchai S, Tran BN, Matsudaira PT, Tassanakajon A & Wangkanont K. Biochemical and structural characterization of a recombinant fibrinogen-related lectin from *Penaeus monodon*. *Sci Rep*. 11 (2021) 29.
 - 32 Liu Y, Su Y, Zhang A & Cui Z. A C-Type Lectin highly expressed in *Portunus trituberculatus* intestine functions in AMP regulation and prophenoloxidase activation. *Antibiotics*. 10 (2021) 541.
 - 33 Baliarsingh S, Sahoo S, Jo YH, Han YS, Sarkar A, Lee YS, Mohanty J & Patnaik BB. Molecular cloning, sequence characterization and expression analysis of C-type lectin (CTL) and ER-Golgi intermediate compartment 53-kDa protein (ERGIC-53) homologs from the freshwater prawn, *Macrobrachium rosenbergii*. *Aquac Int*, 30 (2022) 1011.
 - 34 Sowmiya S, Shanthi R, Athira M & Sivakumar MR. Morphological and phagocytosis involvement of hemocytes

- from the freshwater crab *Barytelphusa cunicularis* (Westwood in Sykes, 1836). *Micron*, 201 (2026) 103941.
- 35 Nawaz N, Wen S, Wang F, Nawaz S, Raza J, Iftikhar M & Usman M. Lysozyme and its application as antibacterial agent in food industry. *Molecules*, 27 (2022) 6305.
 - 36 Dong B & Sun C. Production of an invertebrate lysozyme of *Scylla paramamosain* in *E. coli* and evaluation of its antibacterial, antioxidant and anti-inflammatory effects. *Protein Expr Purif*, 177 (2021) 105745.
 - 37 Egessa R. Antimicrobial peptides from freshwater invertebrate species: potential for future applications. *Mol Biol Rep*, 49 (2022) 9797.
 - 38 Matos GM & Rosa RD. On the silverjubilee of crustacean antimicrobial peptides. *Rev Aquacult*, 14 (2022) 594.
 - 39 Saucedo-Vazquez JP, Gushque F, Vispo NS, Rodriguez J, Gudino-Gomezjurado ME, Albericio F, Tellkamp MP & Alexis F. Marine arthropods as a source of antimicrobial peptides. *Mar Drugs*, 20 (2022) 501.
 - 40 Barreto C, Matos GM & Rosa RD. On the wave of the crustin antimicrobial peptide family: From sequence diversity to function. *Fish Shellfish Immunol Rep*, 3 (2022) 100069.
 - 41 Anju A, Smitha CK, Preetha K, Boobal R & Rosamma P. Molecular characterization, recombinant expression and bioactivity profile of an antimicrobial peptide, Ss-arsin from the Indian mud crab, *Scylla serrata*. *Fish Shellfish Immunol*, 88 (2019) 352.
 - 42 Xie Y, Wan H, Zeng X, Zhang Z & Wang Y. Characterization and antimicrobial evaluation of a new Spgly-AMP, glycine-rich antimicrobial peptide from the mud crab *Scylla paramamosain*. *Fish Shellfish Immunol*, 106 (2020) 384.
 - 43 Yang Y, Chen F, Chen HY, Peng H, Hao H & Wang KJ. A novel antimicrobial peptide scyreprocin from mud crab *Scylla paramamosain* showing potent antifungal and antibiofilm activity. *Front Microbiol*, 11 (2020) 1589.
 - 44 Zdybicka-Barabas A, Stączek S, Kunat-Budzyńska M & Cytryńska M. Innate immunity in insects: The lights and shadows of phenoloxidase system activation. *Int J Mol Sci*, 26 (2025) 1320.
 - 45 Yang W, He Y, Liang H, Lin J, Tran NT, Du Z & Li S. C-type lectin (SpCTL) through its binding to Sp β -integrin regulates antimicrobial peptide synthesis in mud crab (*Scylla paramamosain*) during *Vibrio parahaemolyticus* infection. *Aquac Rep*, 42 (2025) 102804.
 - 46 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Chem*, 72 (1976) 248.
 - 47 Roe JH. The determination of sugar and blood and spinal fluid with anthrone reagent. *J Biol Chem*, 212 (1955) 335.
 - 48 Folch J, Lees M & Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem*, 2261 (1957) 497.
 - 49 Sivakumar MR & Shanthi R. Inhibition and activation of humoral phenoloxidase and their effect on divalent cations in the hemolymph of freshwater prawn *Macrobrachium rosenbergii*. *Int. J. Zool. Anim. Biol*, 6(6) (2023) 000540.
 - 50 Garvey JS, Cremer NE & Sussdorf DH. *Methods in Immunology: A Laboratory Text for Instruction and Research*, 3rd edn. (W. A. Benjamin, Massachusetts, USA), (1977) 545.
 - 51 Hultmark D, Steiner H, Rasmuson T & Boman HG. Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. *Eur J Biochem*, 106 (1980) 7.
 - 52 Holt JG, Krieg NR, Sneath PHA, Staley JT & Williams ST. *Bergey's Manual of Determinative Bacteriology*. 9th edn. Williams and Wilkins, Baltimore, Maryland, USA, (1994).
 - 53 Magaldi S, Mata-Essayag S, De Capriles CH, Perez C, Colella MT, Olaizola C & Ontiveros Y. Well diffusion for antifungal susceptibility testing. *Int. J. Infect. Dis*, 8 (2004) 39.
 - 54 Moruf RO, Saba AO, Chukwu-Osazuwa J & Elegbede IO. Seasonal variation in macro-micronutrient compositions of the flesh and shell of the portunid crab *Callinectes amnicola* from the coastal waters of Southwest Nigeria. *Agric*, 28 (2019) 200.
 - 55 Li X, Yuan Y, Jin M, Wang X, Hu X, Zhao M, Luo J, Xu F, Zhao Y, Jiao L & Zhou Q. Growth performance, antioxidant capacity, tissue fatty acid composition and lipid metabolism of juvenile green mud crab *Scylla paramamosain* in response to different dietary n-3 PUFA lipid sources. *Aquac. Rep*, 19 (2021) 100599.
 - 56 Masuda T. Update on the source of phenoloxidase activity in the hemolymph of kuruma prawn *Marsupenaeus japonicus*. *Fish. Sci*, 87 (2021) 861.
 - 57 Rubeena AS & Preetham E. Antimicrobial properties and phenoloxidase activation of the lectin isolated from kadal shrimp *Metapenaeus dobsoni*. *Fish Shellfish Immunol*, 90 (2019) 118.
 - 58 Goncalves AA & de Oliveira ARM. Melanosis in crustaceans: A review. *LWT*, 65 (2016) 791.
 - 59 Coates CJ & Nairn J. Non-specific electrostatic interactions and the conversion of horseshoe crab haemocyanin into a phenoloxidase. *Dev Comp Immunol*, 169 (2025) 105401.
 - 60 Xu S, Jing M, Liu WY, Dong H, Kong DM, Wang YR, Zhang HH, Yue Z, Li YJ, Jiao F & Xie SY. Identification and characterization of a novel L-type lectin (MjLTL2) from kuruma shrimp *Marsupenaeus japonicus*. *Fish Shellfish Immunol*, 98 (2020) 354.
 - 61 Dilna C, Prasanth GK, Ghufraan MS, Soni P, Kanade SR & Duddukuri GR. Purification and characterization of a hemocyanin with lectin-like activity isolated from the hemolymph of speckled shrimp *Metapenaeus monoceros*. *Biochimie*, 206 (2023) 36.
 - 62 Priyatharshini JC, Rose MRB & Glory PTA. Agglutinin in the hemolymph of *Barytelphusa cunicularis*. *J Emerg Technol Innov Res*, 5 (2018) 210.
 - 63 Ramaraj P, Bhuvragavan S, Sruthi K, Ranjana B, Subramanian KA, Stella C & Janarthanan S. N-acetyl-D-glucosamine-binding lectin (PrLec) from the serum of healthy blue swimming crab *Portunus reticulatus* agglutinates Gram-positive (*Micrococcus luteus*) and Gram-negative (*Escherichia coli*) bacteria. *Aquac Res*, 53 (2022) 4893.
 - 64 Elayabharathi T, Mary JVJ & Bai SMM. Characterization of a novel O-acetyl sialic acid specific lectin from the hemolymph of the marine crab *Atergatis integerrimus* (Lamarck, 1818). *Fish Shellfish Immunol*, 106 (2020) 1131.
 - 65 Lu J, Yu Z, Mu C, Li R, Song W & Wang C. Characterization and functional analysis of a novel C-type

- lectin from the swimming crab *Portunus trituberculatus*. *Fish Shellfish Immunol*, 64 (2017) 185.
- 66 Zhang W, Zhang Z, Mu C, Li R, Ye Y, Zhang H, Song W, Shi C, Liu L & Wang C. Molecular cloning, characterization and expression of a C-type lectin from *Scylla paramamosain*, which might be involved in the innate immune response. *Fish Shellfish Immunol*, 93 (2019) 251.
- 67 Bai SMM & Rose MRB. Purification and characterization of hemolymph lectin of freshwater crab *Lamella lamellifrons* (Alcock, 1909). *Ann Trop Med Public Health*, 23 (2020) 611.
- 68 Wei X, Wang L, Sun W, Zhang M, Ma H, Zhang Y, Zhang X & Li S. C-type lectin B (SpCTL-B) regulates the expression of antimicrobial peptides and promotes phagocytosis in mud crab *Scylla paramamosain*. *Dev Comp Immunol*, 84 (2018) 213.
- 69 Cao XT, Pan XY, Sun M, Liu Y & Lan JF. Hepatopancreas-specific lectin participates in the antibacterial immune response by regulating the expression of antibacterial proteins. *Front Immunol*, 12 (2021) 679767.
- 70 Viana JT, dos Santos Rocha R & Maggioni R. Structural and functional diversity of lectins associated with immunity in the marine shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol*, 129 (2022) 152.
- 71 Fujii Y, Fujiwara T, Koide Y, Hasan I, Sugawara S, Rajia S, Kawsar SM, Yamamoto D, Araki D, Kanaly RA, Ogawa Y, Fujita H & Ozeki Y. Internalization of a novel huge lectin from *Ibacus novemdentatus* induces apoptosis of mammalian cancer cells. *Glycoconj J*, 34 (2017) 85.
- 72 Cai S, Zhang Y, Wu F, Wu R, Yang S, Li Y & Xu Y. Identification and functional characterization of a C-type lysozyme from *Fenneropenaeus penicillatus*. *Fish Shellfish Immunol*, 88 (2019) 161.
- 73 Xie JW, Cheng CH, Ma HL, Feng J, Su YL, Deng YQ & Guo ZX. Molecular characterization, expression and antimicrobial activities of a C-type lysozyme from the mud crab *Scylla paramamosain*. *Dev Comp Immunol*, 98 (2019) 54.
- 74 Divya M, Vaseeharan B, Anjugam M, Iswarya A, Karthikeyan S, Velusamy P, Govindarajan M, Alharbi NS, Kadaikunnan S, Khaled JM & Vagvolgyi C. Phenoloxidase activation, antimicrobial and antibiofilm properties of β -glucan binding protein from *Scylla serrata* crab hemolymph. *Int J Biol Macromol*, 114 (2018) 864.
- 75 Gnaneswari MD, Sharma S, Ghosh C, Tyagi S & Regina T. Identification and partial characterization of antibacterial proteins in field crab *Paratelphusa hydrodromous*. *J Environ Biosci*, 39 (2025) 55.
- 76 Jayanthi S, Ishwarya R, Anjugam M, Iswarya A, Karthikeyan S & Vaseeharan B. Purification, characterization and functional analysis of the immune molecule lectin from the haemolymph of blue swimmer crab *Portunus pelagicus* and their antibiofilm properties. *Fish Shellfish Immunol*, 62 (2017) 227.
- 77 Anbuhezian R, Ravichandran S, Rajan DK, Tilivi S & Devi SP. Identification and functional characterization of antimicrobial peptide from the marine crab *Dromia dehaani*. *Microb Pathog*, 125 (2018) 60.
- 78 Zhang XW, Man X, Huang X, Wang Y, Song QS, Hui KM & Zhang HW. Identification of a C-type lectin possessing both antibacterial and antiviral activities from red swamp crayfish. *Fish Shellfish Immunol*, 77 (2018) 22.