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Bio-mining drugs from the sea: High antibiofilm properties of haemocyanin purified from the haemolymph of flower crab *Portunus pelagicus* (L.) (Decapoda: Portunidae)

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ABSTRACT

Marine organisms are an outstanding source of molecules with wide biological activities. In this research, we investigated the antimicrobial protein haemocyanin isolated from the haemolymph of flower crab (*Portunus pelagicus*) using gel filtration chromatography. Purified *P. pelagicus* haemocyanin (*Pp*-Hc) was subjected to SDS-PAGE analysis and the fraction containing *Pp*-Hc ran as a distinct band, its molecular weight was 78 kDa. The *Pp*-Hc was characterized through HPLC, XRD, CD and FTIR analyses. The antibacterial potential of *Pp*-Hc was evaluated against five Gram-positive and ten Gram-negative bacteria through the agar well diffusion method, which clearly revealed the high bactericidal activity of *Pp*-Hc. Furthermore, *Pp*-Hc was studied for its antibiofilm activity against five biofilm-forming Gram-negative bacteria, i.e., *Vibrio alginolyticus*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, and *Proteus vulgaris*. Confocal laser scanning microscopy (CLSM) and light microscopy evidenced significant antibiofilm properties of *Pp*-Hc at 100 μg/ml concentration. Based on exopolysaccharide (EPS) quantification and cell surface hydrophobicity (CSH) index, the antibiofilm action of *Pp*-Hc was linked to its ability to reduce EPS production and increase cell surface hydrophobicity. The pathogen recognition mechanism of purified *Pp*-Hc showed a wide spectrum of binding activities, leading to high toxicity against both Gram-positive and Gram-negative bacterial species. Furthermore, the purified *Pp*-Hc showed high antifungal activity against the pathogen *Candida albicans*. Haemocyanin is not only involved in transporting oxygen, it has a relevant function in the flower crab immune system. This work adds useful knowledge for aquaculture concerns dealing with microbial pathogen control. Overall, our results outlined the high potential of purified *Pp*-Hc as a starting material to develop new and effective antimicrobial drugs for aquaculture purposes.

1. Introduction

The flower crab, *Portunus pelagicus* (L.) (Decapoda: Portunidae) is an edible crab, which inhabits the intertidal zones all over the Indo-Pacific region, therefore playing a chief role in the marine ecosystems (Lestang et al., 2003; Kunsook and Dumrongrojwatthana, 2017). It is a vital species in aquaculture industries. However, in the last few years, crab farming has been threatened by the increase of infectious diseases due to bacterial, fungal and viral pathogens (Destoumieux-Garzon et al., 2001). Current microbiology and parasitology are facing the growing lack of effective antibiotic drugs. Indeed, antibiotics must be used prudently to treat humans and animals, since their prolonged overuse led to the fast development of resistance in targeted organisms (Davies and Davies, 2010). Antibiotic resistance now represents a serious

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concern in both developed and developing nations (Hernández Serrano, 2005; Ventola, 2013). Eco-friendly solutions to fight the growth of multiple antibiotic resistance, resistance transfer to pathogenic bacteria, and reduced efficacy of antibiotic treatment for diseases caused by resistant pathogens, are of timely importance nowadays (Levy, 2002; Ishwarya et al., 2016; Anjugam et al., 2016; Iswarya et al., 2017; Jayanthi et al., 2017). A way to tackle the problem is to shift towards the use of compounds with multiple mechanisms of action for limited periods of time, to avoid prolonged exposures of a given organisms to the same toxic molecules, which in turn leads to grow of resistance to those drugs (Benelli et al., 2017). Therefore, developing new antibiotics isolated from natural sources can help to prevent losing fights against infectious agents.

Marine organisms represent an outstanding source of molecules with wide biological activities (Torres et al., 2014; Debbab et al., 2010), and numerous bioactive compounds have also been isolated from marine sources such as ziconotide from *Conus* snails (Bulaj et al., 2003), marthiapeptide A from *Marinactinospora thermotolerans* (Zhou et al., 2012a), capoamycin from *Streptomyces fradiae* (Xin et al., 2012), ageloxime B from *Agelas mauritiana* (Yang et al., 2012), Sp-ALF, antilipopolysaccharide factor and scygonadin from *Scylla paramamosain* (Liu et al., 2012; Peng et al., 2012), and antilipopolysaccharide factor (PtALF7) from *Portunus trituberculatus* (Liu et al., 2013), just to cite few examples. Recently, numerous researches focused on crustacean immunity to manage microbial diseases, to lead towards enhanced output in aquaculture industries.

Haemocyanins are among the most important copper containing proteins, representing up to 95% of the hemolymph of crustaceans (Sellos et al., 1997; Jayasree, 2001; Zheng et al., 2016; Fredrick and Ravichandran, 2012). Hemocyanins are extracellular negatively charged proteins involved in numerous physiological functions, like protein storage, osmoregulation, oxygen transport and enzyme activities (Paul and Pirow, 1997; Decker et al., 2007; Markl, 2013). They contribute to growth of homeostasis and invertebrates' defenses against pathogens (Coates and Nairn, 2014). They can perform as antiviral agents against a variety of viruses. It has been pointed out that they holdup the infection of white spot syndrome virus (WSSV) *in vivo* (Lei et al., 2008). Also, haemocyanin can respond with anti-human Ig as an antigen (Zhang et al., 2006). The antiviral properties of haemocyanin are not limited to invertebrate systems, and predominantly those purified from mollusks. Indeed, they can induce potent immunostimulatory responses in mammalian hosts, and show notable anti-viral (Nesterova et al., 2011), anti-cancer (Dolashka et al., 2011; Lammers et al., 2012), anti-parasitic (Guo et al. 2009 & 2011) and therapeutic potential (Dolashka-Angelova et al., 2008; Becker et al., 2009; Zanjani et al., 2014).

Previous researches reported that haemocyanins can act as antimicrobial agents, in the form of activators and as source of antimicrobial proteins (Kawabata et al., 1995; Velayutham and Munusamy, 2016). Very recently, we have described that the haemocyanin (74 kDa) can be purified from the haemolymph of *Fenneropenaeus indicus*, showing remarkable antibiofilm activity on bacterial pathogens. The antibacterial and antibiofilm activity of haemocyanin is not obvious. It should be investigated if the observed activity is due to triggered changes EPS production, biofilm inhibition, agglutination of bacteria, bactericidal activity, and/or antibiofilm action.

Therefore, in this research, we investigated the antimicrobial protein haemocyanin isolated from the haemolymph of flower crab (*Portunus pelagicus*) using gel filtration chromatography. Purified *P. pelagicus* haemocyanin (*Pp*-Hc) was subjected to SDS-PAGE analysis. The antibacterial activity of *Pp*-Hc was evaluated against both Gram-positive and -negative bacteria through the agar well diffusion method. Furthermore, *Pp*-Hc was studied for its antibiofilm activity against five biofilm-forming Gram-negative bacteria. Confocal laser scanning microscopy (CLSM) and light microscopy were used to evaluate the antibiofilm properties of *Pp*-Hc. Exopolysaccharide (EPS) quantification and cell surface hydrophobicity (CSH) index shed light on the mechanism of action of this molecule against microbial pathogens. In addition, the purified *Pp*-Hc was tested for antifungal activity on the pathogen *Candida albicans*.

2. Materials and methods

2.1. Crabs

The flower crabs, *P. pelagicus* were collected from Mandapam, Ramnad District, Tamil nadu, India (9.2770° N, 79.1252° E) and kept in large FRP (Fiberglass reinforced plastics) tanks containing 300 l water with (salinity: 28 g L ± 1, temperature: 27 °C ± 4 °C, dissolved oxygen ≥ 5.5 mg L^{-1}). Before sacrificing, the healthy crabs among the normal weight of 150 g were stored and maintained for 3 days.

2.2. Bacterial and fungal strains

The antibacterial activity of crab-borne haemocyanin was studied against both Gram-positive bacteria *Bacillus licheniformis* (HM235407), *Bacillus pumilis* (HQ693273), *Bacillus thuringiensis* (KC465903), *Bacillus lentus* (HQ116443), *Enterococcus faecalis* (HQ693279) and Gram-negative *Vibrio alginolyticus* (ATCC 17749), *Vibrio parahaemolyticus* (HQ693275), *Vibrio harveyi* (HQ693276) *Pseudomonas aeruginosa* (HQ693274), *Proteus vulgaris* (HQ116441), *Escherichia coli* (ATCC) *Citrobacter murliniae* (KC465906), *Citrobacter freundii* (KC465905), *Morganella morganii* (KC465904), *Citrobacter amalonaticus* (HQ3830347), as well as on the fungus *Candida albicans* (MTCC – 7315).

2.3. Haemolymph collection

Haemolymph was withdrawn from the right chelate leg using a 25-gauge sterile needle. To escape from the risk of coagulation, haemolymph was stabilized with same quantity of anticoagulant solution (Dextrose = 10.25 g, Tris sodium citrate = 4 g, citric acid = 0.28 g, NaCl = 2.10 g, distilled H_2O = 500 ml). The anticoagulant stabilized haemolymph was centrifuged at 7000 × *g* for 10 min, to separate the crab haemocytes from haemolymph.

2.4. Purification of haemocyanin from P. pelagicus

The haemocyanin was purified following the method by Ishwarya et al. (2016) with minor changes. To purify haemocyanin from the supernatant of haemolymph, gel filtration chromatography was carried out at 4 °C; 20 ml of TBS supernatant was run through Sephadex G-100 matrix, pre-equilibrated with TBS/$CaCl_2$ buffer. Subsequently, the column was rinsed with washing buffer, the resultant eluent containing haemocyanin was collected, and stored at −20 °C for further use.

2.5. Characterization of purified haemocyanin from P. pelagicus

2.5.1. SDS PAGE analysis

SDS PAGE was carried out by 12% separating gel and 4% stacking gel following the Laemmli (1970) method. The haemocyanin molecular mass was identified by comparing the respective mobility with standard protein marker (Hi Media, Bangalore, India).

2.5.2. XRD and FTIR spectroscopy

Purified *Pp*-Hc was analyzed by XRD (XRD, Scintag-SDS 2000) at 40 kV/20 mA, using a continuous scanning 2θ mode. *Pp*-Hc average grain size and shape were estimated using Scherrer's formula [d = (0.9ʎ θ cosθ), where d was the diameter of *Pp*-Hc, ʎ is the wavelength of the X-ray source, and β is the angular FWHM of the XRD peak at the diffraction angle (θ). For FTIR spectroscopy, *Pp*-Hc (1–50 μl) was placed in a thermostated cell fixed by CaF_2 windows. Spectra of biomolecules

were measured at a resolution of 4 cm^{-1}.

2.5.3. Circular dichroism and High performance liquid chromatography

The secondary structure of the *Pp*-Hc was analyzed by CD spectrum, value ranged between from 250 to 190 nm at a speed of 50 nm/min. A 1-mm-path-length quartz cuvette was used for the measurements. *Pp*-Hc samples were measured at 30–40 μl in 20 mM Tris-HCl-20 mM NaCl, pH 7.4, with or without 20 mM of SDS. The consistency of *Pp*-Hc was evaluated using a reversed-phase HPLC (Zorbax Bio-series GF-250, Du Pont, and Willington, DE, USA) C_{18} column (7.8 mm × 30 cm) with a linear gradient among 0.05% trifluoroacetic acid in water and 0.052% trifluoroacetic acid in 80% acetonitrile. The column was standardized by reference proteins for molecular mass evaluation in matching conditions.

2.6. Antimicrobial activity

2.6.1. Antibacterial activity test

To screen the antibacterial potent of *Pp*-Hc, agar well diffusion method was employed. Bacterial suspensions were evenly spread with support of sterilized cotton swab on sterile LB agar plate. Seven millimeter in diameter wells were cut using a sterile cork borer, each well possessed 20 mm distance from one another. To the wells, the purified *Pp*-Hc at different concentrations (50 and 100 μg/ml) was loaded and incubated at 37 °C for 24 h. Inhibition of bacterial growth was monitored measuring the clear zone formed around the well in mm using a ruler. Each suspension was tested in at least three independent experiments.

2.6.2. Minimum inhibitory concentration (MIC)

The MIC at which a purified haemocyanin exhibited antibacterial activity was determined by microtiter plate assay. Nutrient broth 250 μl was poured and the microorganisms were inoculated into the wells. 10 μl, 25 μl, 50 μl, 75 μl and 100 μl of *Pp*-Hc were added to the wells and incubated at 37 °C for 24 h. Following the incubation, OD was measured at 600 nm using the Elisa plate reader. All the experiments were carried out in three replicates.

2.6.3. Bacteria growth inhibition assay

The impact of *Pp*-Hc on the growth of bacteria was evaluated using a growth curve assay. Gram negative bacteria *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus*, *P. aeruginosa* and *P. vulgaris* were tested. 1 ml of overnight culture was transferred to test tube and incubated along with purified haemocyanin at 3 different concentrations for growth and turbidity analysis. Microbial growth was judged from the turbidity change monitored every 2 h interval with continuous shaking (200 rpm) at 37 °C, and OD 600 was calculated up to 8 h. Growth curves were prepared based on the data from 3 replicates for each tested dose.

2.6.4. In vitro killing assay

The ability of haemocyanin to kill bacteria was screened through *in vitro* killing assay followed by Jin et al. (2012) with small changes. Overnight Gram negative bacterial culture were centrifuged for 5 min at 6000 × *g* and the resultant pellet was rinsed and suspended with TBS buffer showing approximately 0.1 nm value at 600 OD. Bacterial suspensions were mixed with different concentrations of *Pp*-Hc in challenged wells whereas in unchallenged wells, TBS buffer replace *Pp*-Hc. The above said suspensions were incubated at 37 °C for 1 to 2 h and the lethal action of haemocyanin was monitored under inverted light microscope.

2.6.5. EPS quantification assay

EPS quantification was performed following the method by Vinoj et al. (2015) with minor changes. The species *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus*, *P. aeruginosa*, and *P. vulgaris* were added to LB broth supplemented with or without *Pp*-Hc at 50 and 100 μg/ml, and

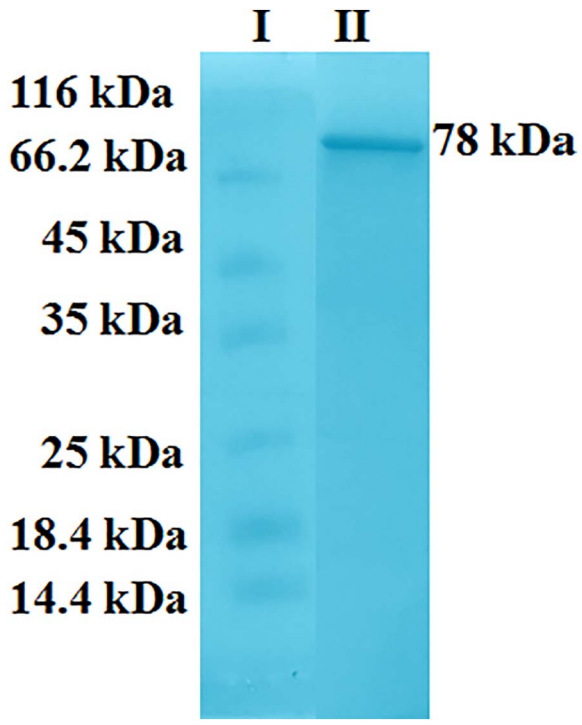

Fig. 1. Haemocyanin purified from the haemolymph of *Portunus pelagicus*. Lane I: Protein marker. Lane II: Purified haemocyanin, molecular weight: about 78 kDa.

then incubated for 24 h at 37 °C. Then, the bacterial cells were rinsed with PBS buffer, and the eluent fraction was incubated again with 50 and 100 μg/ml of *Pp*-Hc. After incubation, spectrophotometric analysis was done for EPS quantification.

The main values and the standard deviation were calculated from the data acquired with triplicate experiments.

2.6.6. Quantification of CSH by MATH assay

For quantification of CSH, the bacteria were cultured in LB and harvested by centrifugation at 6000 rpm for 5 min at 25 °C, rinsed, resuspended in sterile deionized water and adjusted to an OD at 600 nm. The *Pp*-Hc and toluene (1 ml) were added to 2 ml of the cell suspension (A600) in a test tube. Then, after vortexing for 1 min, the cell suspensions were incubated at 37 °C overnight, lastly then OD was measured at 600 nm. All the experiments were performed in triplicates.

2.6.7. Biofilm quantification assay

The impacts of purified Pp-Hc on the biofilm-forming Gram-negative bacteria were tested on 24-well plates. An overnight culture (10^7 CFU/ml) of Gram-negative bacteria was used. Glass pieces were incubated for 24–72 h at 37 °C and stained by 0.4% crystal violet (CV) for 10 min. Then 1 ml of absolute ethanol was used for the wells were allowed to dry before solubilization of the CV and OD was measured at 570 nm. Biofilm quantification was done in triplicates wells in three replicate experiments.

2.6.8. Antibiofilm assay

The antibiofilm activity of purified *Pp*-Hc was tested on biofilm-forming Gram-negative bacteria and the fungus *C. albicans*. 1.5 ml of the culture were distributed on the wells of microtiter plate having glass pieces, then supplemented with *Pp*-Hc (50 and 100 μg/ml) and the plate was incubated at 37 °C for 24 h in static condition. Therefore, the glass pieces were rinsed with 0.01 M PBS to remove unadhered planktonic cells. The glass slides were stained with 0.4 % crystal dye and observed under bright field microscope at 40× magnification. As carried out for CLSM analysis, the biofilms formed on the glass slides in the presence or

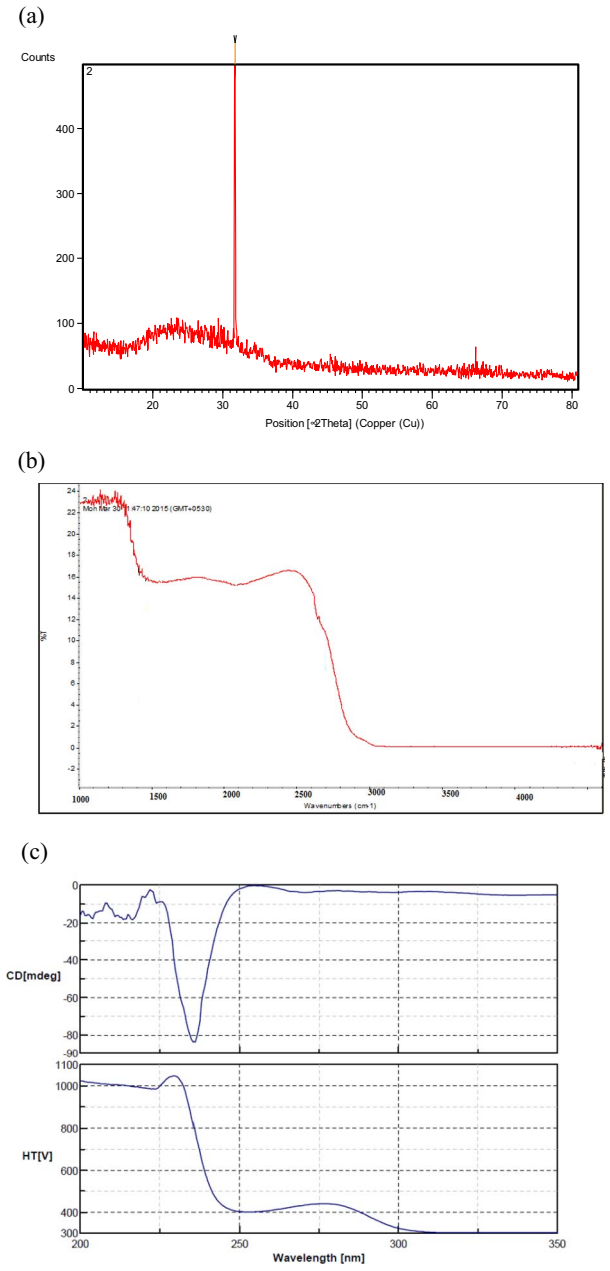

Fig. 2. (a) Crystalline surface and lattice arrangement of molecules in the *P. pelagicus* purified haemocyanin analyzed through XRD. (b) Fourier transform infrared spectroscopic (FTIR) spectrum of *P. pelagicus* purified haemocyanin. (c) Circular dichroism spectrum of *P. pelagicus* purified haemocyanin.

not of purified *Pp*-Hc (50 and 100 μg/ml) were stained with 0.4 % of acridine orange and then analyzed via CLSM microscopy.

2.6.9. Data analysis

Data concerning the *Pp*-Hc-triggered growth inhibition, toxicity, EPS production and CSH in microbial pathogens were analyzed using analysis of variance (ANOVA) followed by Tukey's HSD test. A *P*-value of 0.05 was used as threshold to evaluate significant differences between means.

3. Results

3.1. Purification of haemocyanin from P. pelagicus

Pp-Hc was purified from the haemolymph of *P. pelagicus* by gel filtration chromatography. *Pp*-Hc purity and molecular mass was studied through gel electrophoresis. Fig. 1 shows a single band of 78 kDa in 12 % SDS-PAGE under reducing conditions.

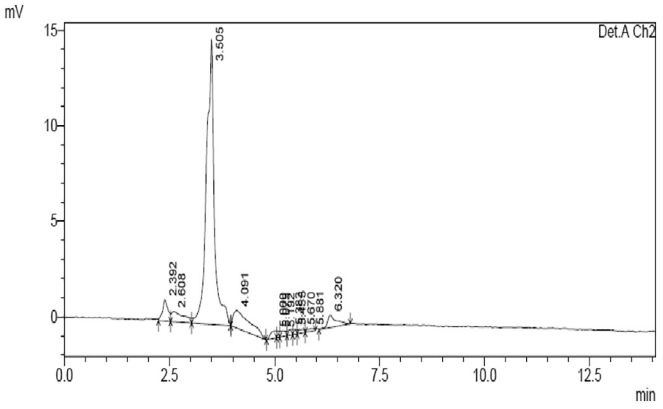

Fig. 3. HPLC analysis of purified haemocyanin isolated from the haemolymph of *P. pelagicus*.

Table 1
Antibacterial activity of crab-borne purified haemocyanin against 15 bacterial species.

No.	Bacteria	Zone of inhibition (mm)	
		50 μg/ml of purified *Pp*-Hc	100 μg/ml of purified *Pp*-Hc
Gram-positive bacteria			
1	*Bacillus licheniformis*	4.3 ± 0.5 a	6.5 ± 0.2 a
2	*Bacillus pumilis*	–	–
3	*Bacillus thuringiensis*	–	–
4	*Bacillus lentus*	5.0 ± 0 b	7.0 ± 0.9 a
5	*Enterococcus faecalis*	–	–
Gram-negative bacteria			
6	*Vibrio alginolyticus*	17.0 ± 1.0 ef	24.0 ± 1.0 e
7	*Vibrio parahaemolyticus*	16.5 ± 1.2 de	26.5 ± 0.6 f
8	*Vibrio harveyi*	15.3 ± 0.3 d	20.3 ± 0.7 d
9	*Pseudomonas aeruginosa*	18.2 ± 0.6 f	26.3 ± 1.6 f
10	*Proteus vulgaris*	16.2 ± 0.5 e	21.2 ± 0.9 d
11	*Escherichia coli*	8.3 ± 0.4 c	10.3 ± 0.4 b
12	*Citrobacter murliniae*	9.03 ± 0.6 c	14.0 ± 0 c
13	*Citrobacter freundii*	7.3 ± 0.6 bc	12.4 ± 0.6 b
14	*Morganella morganii*	8.6 ± 0.5 c	15.2 ± 1.2 c
15	*Citrobacter amalonaticus*	9.5 ± 1.6 c	14.7 ± 0.6 c

Values are means ± SD of 3 replicates.
Within a column, different letters indicate significant differences (ANOVA, Tukey's HSD, $P < 0.05$).

3.2. XRD and FTIR spectroscopy

In XRD analysis, *Pp*-Hc showed a single peak at 32.5° indexing (103) planes, thus underlining the *Pp*-Hc crystalline nature. The lattice constant calculated from this pattern was a = 2.8175 Å. Data agreed with the database of Joint Committee on Powder Diffraction Standards (JCPDS) file no. #88-0110 (Fig. 2a). FTIR spectrum of *Pp*-Hc displayed amide I peak within the spectral range 1620–1700 cm^{-1} consequent to the β-sheet structure, and amide II peaks showing an α-helical conformation at 1650–1700 cm^{-1} (Fig. 2b).

3.3. CD and HPLC

The secondary structure of *Pp*-Hc was predicted by CD scanning between 200 and 350 nm (Fig. 2c). Signals acquired at 210–230 nm were because of the weak peptide bond. The broad n-π transition was centered around 210 nm, while an intense p-p transition was found at 190 nm. The CD spectrum of *Pp*-Hc showed a broad negative minimum

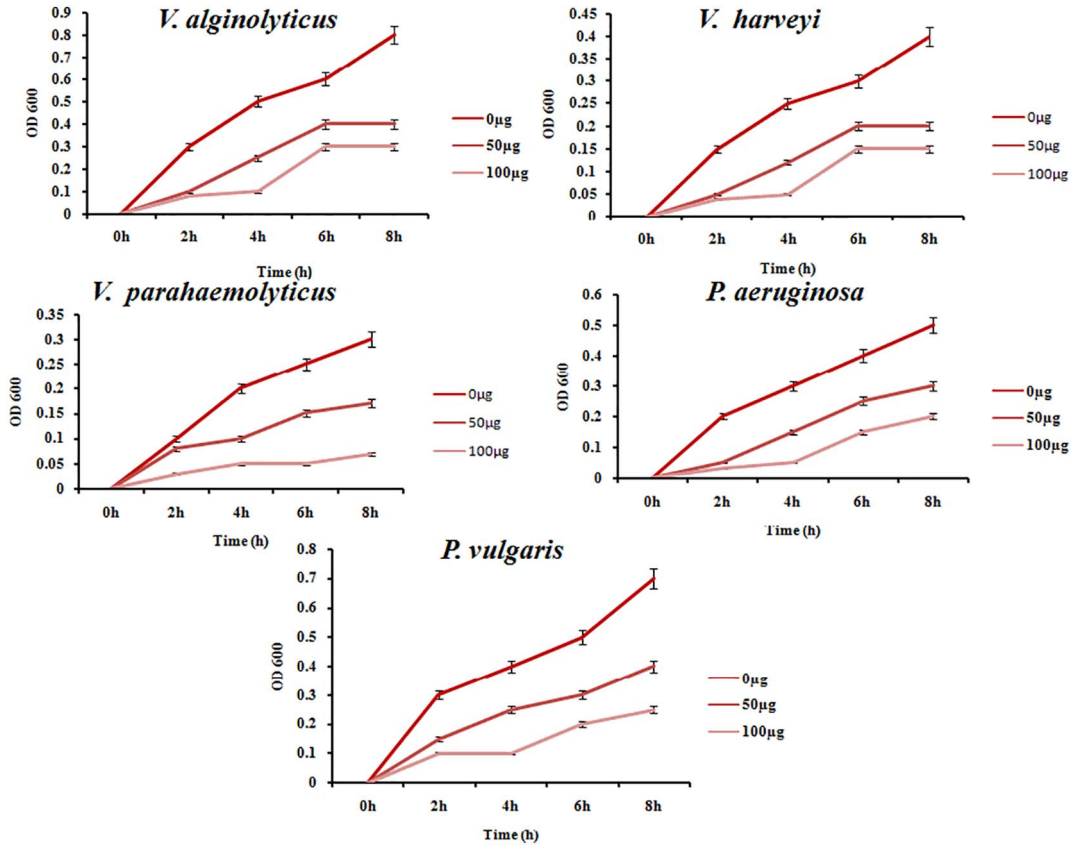

Fig. 4. Growth inhibitory activity of purified haemocyanin isolated from the haemolymph of *P. pelagicus* against selected bacterial pathogens. Results were expressed as mean values ± SD of three independent data.

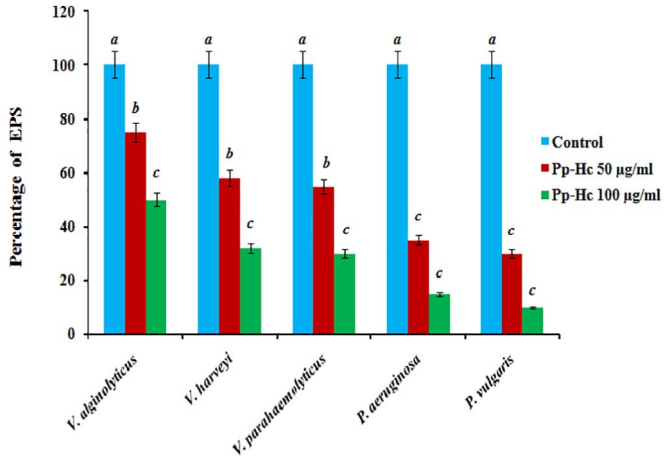

Fig. 5. EPS inhibition (%) in Gram-negative bacteria treated with purified haemocyanin isolated from the haemolymph of *P. pelagicus*. Each bar indicates the mean ± SD of three independent experiments. Within each species, columns with different letters indicate the significant differences between treatments (ANOVA, Tukey's HSD test, $P < 0.05$).

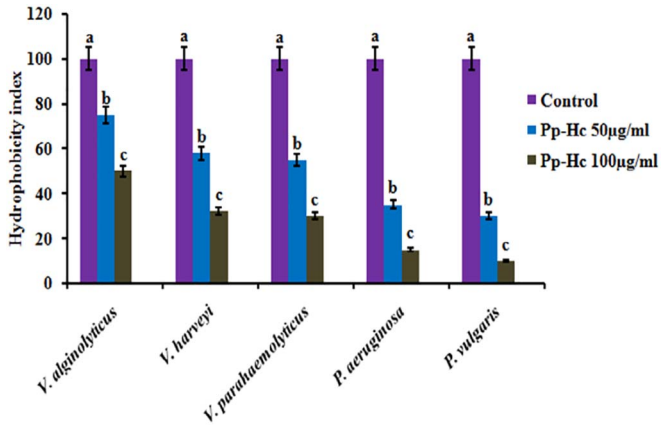

Fig. 6. Effect of purified haemocyanin isolated from the haemolymph of *P. pelagicus* on the cell surface hydrophobicity (CSH) index of Gram negative bacteria. Results were shown as mean values ± SD of three replicates. Within each species, columns with different letters indicate the significant differences between treatments (ANOVA, Tukey's HSD test, $P < 0.05$).

at 200 nm and a crossover at 195 nm. Low positive ellipticity values < 200 nm suggested the occurrence of unordered segments in *Pp*-Hc. The broad negative minimum enlarging from 200 to 350 nm was linked to the occurrence of ß-sheet structure. The reversed phase HPLC with C_{18} column, besides ascertaining the *Pp*-Hc, showed a distinct prominent peak with a retention time of 3.5 min (Fig. 3).

3.4. Antibacterial activity

The antibacterial activity of *Pp*-Hc was assessed by agar well diffusion method against 5 Gram-positive species, i.e., *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus thuringiensis*, *Bacillus lentus*, *Enterococcus faecalis*, and ten Gram-negative species, i.e., *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Escherichia coli*, *Citrobacter amalonaticus*, *Citrobacter murliniae*, *Citrobacter freundii*, and *Morganella morganii*. Results showed broad-spectrum antibacterial activity, considered as the diameter of the zone of inhibition in agar well diffusion method (Table 1). The maximum zone of inhibition ($P < 0.01$) was shown by *V. parahaemolyticus* and *P. aeruginosa*, while no activity was noted against *B. pumilus*, *B. thuringiensis* and *E. faecalis*.

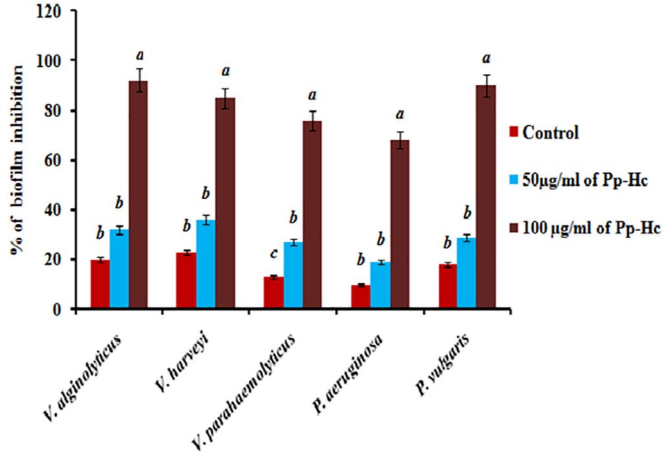

Fig. 7. Impact of purified haemocyanin isolated from the haemolymph of *P. pelagicus* on biofilm formation in Gram-negative bacteria. The results are represented as mean ± SD of three independent experiments. Within each species, columns with different letters indicate the significant differences between treatments (ANOVA, Tukey's HSD test, $P < 0.05$).

3.5. Minimum inhibitory concentration (MIC)

The MIC values of purified *Pp*-Hc dispersed in the microtiter plate are summarized in the Supplementary Online Material Fig. S1, as 24 h OD values of the microbial growth. With the enhancement of tested concentration of purified *Pp*-Hc tested, the final bacterial concentration was significantly reduced ($P < 0.05$). When the concentration was 50 µg/ml, the growth of Gram-negative pathogens was completely inhibited (Supplementary Online Material Fig. S1).

3.6. Bacterial growth inhibition

To find out the antibacterial activity of *Pp*-Hc, its inhibition effect on the growth of Gram negative bacterial cells was examined. The purified *Pp*-Hc inhibited all tested bacteria, if compared with TBS. In addition, the bacterial growth was a little suppressed by 50 µg/ml *Pp*-Hc, and strongly inhibited when the concentration of *Pp*-Hc was increased to 100 µg/ml (Fig. 4).

3.7. Estimation of EPS production

Gram-negative *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus*, *P. aeruginosa* and *P. vulgaris* bacteria were treated with *Pp*-Hc to analyze the effect of *Pp*-Hc on EPS production. Biofilm produced by Gram-negative bacteria was quantified by spectrophotometric analysis. EPS decreased when increasing concentration of tested *Pp*-Hc to 100 µg/ml (Fig. 5). The *Pp*-Hc at the concentration of 50 µg/ml led to reduced EPS quantity in *V. alginolyticus* (25 %) *V. harveyi* (30 %), *V. parahaemolyticus* (40 %), *P. aeruginosa* (50 %), and *P. vulgaris* (75 %).

3.8. Hydrophobicity assay

Cell surface hydrophobicity (CSH) act as a vital part in biofilm formation and adhesion. The consequence of single treatment with *Pp*-Hc at its bacterial inhibitory concentration (BIC) on CSH was calculated. We observed a significant difference in the hydrophobicity index in purified *Pp*-Hc treated samples over values noted in the control (Fig. 6).

3.9. In vitro killing assay

The binding activity of purified *Pp*-Hc induced mortality of bacterial pathogens. Here, we incubated Gram-negative bacteria with purified *Pp*-Hc. The results in terms of microbial aggregation were monitored through light microscopy (Fig. 8). The purified *Pp*-Hc were able to induce aggregation in Gram-negative bacteria at the concentration of 100 µg/ml, while there was only little inhibition testing 50 µg/ml of *Pp*-Hc.

3.10. Biofilm quantification assay

The biofilm formation was monitored by microtiter plates assay using crystal violet dye and measured spectrophotometrically. A calibration curve was carried on Gram-negative bacteria testing two concentrations of purified *Pp*-Hc with control without purified *Pp*-Hc, to quantify biofilm thickness. After 12 h of treatment, samples treat with 50 and 100 µg/ml of *Pp*-Hc exhibited a significant reduction ($P < 0.05$) in biofilm formation when compared to control (Fig. 7).

3.11. Antibiofilm assay

The purified *Pp*-Hc was evaluated on the above-mentioned Gram-negative bacteria. The quantification of biofilm biomass was done by visualizing the biofilm architecture under light and CLSM microscopy, with the staining of crystal violet and acridine orange, respectively. The findings observed a fine developed biofilm growth of tested bacteria in the control slides whereas, bacterial biofilm growth was inhibited post-treatment with *Pp*-Hc. The results confirmed a loss of biofilm architecture in the tested biofilm-forming bacteria (Fig. 9), indicating that *Pp*-Hc effectively inhibited the formation of biofilm. Besides, *Pp*-Hc also express significant antifungal activity ($P < 0.01$) against *C. albicans* (Fig. 10).

4. Discussion

As crustacean oxygen transporter, haemocyanin is involved in the innate defense mechanisms of crustaceans by stimulating enzyme cascade reaction (Terwilliger et al., 1999, Adachi et al., 2005, Coates and Decker, 2017). Currently, about 50 different haemocyanins have been purified and characterized from different crustaceans, insects, chelicerates, myriapods, and onychophorans (Giomi and Beltramini, 2007). Within its superfamily, which poses large groups of proteins, haemocyanin can display different molecular masses (Burmester, 2001).The purified haemocyanins have two distinct subunits, which have been reported in different crustaceans, such as the Kuruma shrimp *Marsupenaeus japonicus* (67 and 77 kDa) (Sivagamavalli and Vaseeharan, 2015), the Pacific white shrimp *Litopenaeus vannamei* (75 and 82 kDa) (Figueroa-Soto et al., 1997), the Northern white shrimp *Litopenaeus setiferus* (77 and 82 kDa) (Brouwer et al., 1978), the mud crab *Scylla serrata* (70-80 kDa) (Yan et al., 2011) and the giant isopod *Bathynomus giganteus* (70 and 72 kDa) (Pless et al., 2003); single bands were reported in the mud crab *Scylla olivacea* (400 kDa) (Chen et al., 2007), as well as in the Indian white shrimp *Fenneropenaeus indicus* (74 kDa) (Ishwarya et al., 2016). In our study, the haemolymph was collected from *P. pelagicus* to isolate *Pp*-Hc by employing gel filtration chromatography. SDS-PAGE analysis was carried out to assess the molecular mass of purified *Pp*-Hc, which resulted to be 78 kDa. Through our report, *Pp*-Hc purity was confirmed via SDS-PAGE and HPLC analysis. The results confirmed the purity by way of single band and high peak at retention time of 3.505 min. Recently, marine sources receive significant attention for various biomedical purposes, including the development of antibacterial drugs of pharmaceutical importance (Jiang et al., 2011).

In the present study, we illustrate the purification and characterization of haemocyanin molecule from the haemolymph of *P. pelagicus*, showing a wide range of antibacterial and antibiofilm activities. Earlier research agrees with our results. Indeed, in other arthropods, haemocyanin-derived peptides exhibit broad-spectrum antimicrobial properties. This has been showed in several species, including *Penaeus*

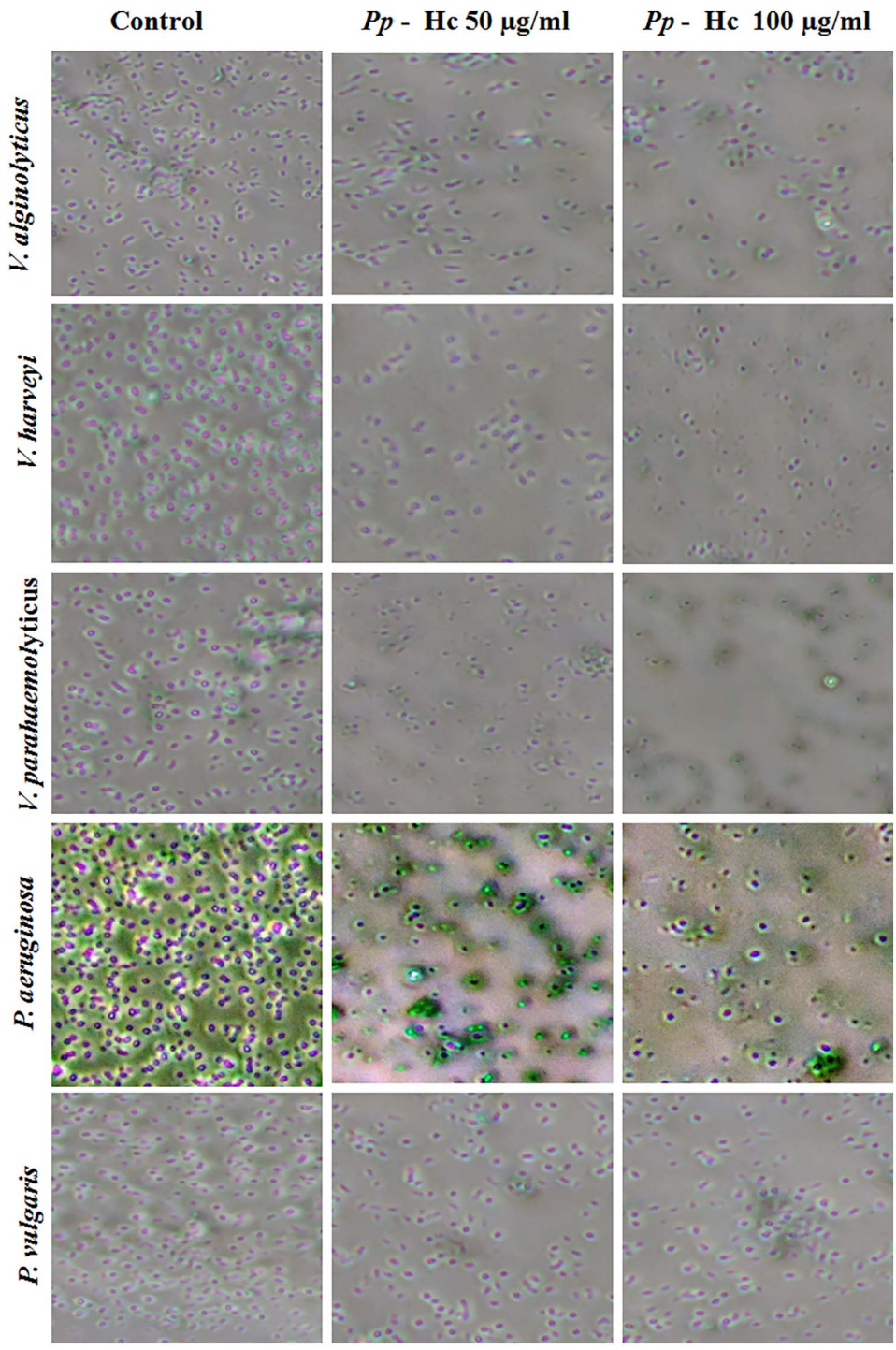

Fig. 8. Effect of the purified haemocyanin isolated from the haemolymph of *P. pelagicus* on the biofilm growth of Gram-negative bacteria observed under light microscopy.

stylirostris (Destoumieux-Garzon et al., 2001), *Pacifastacus leniusculus* (Lee et al., 2003), the spider *Acanthoscurria rondoniae* (Riciluca et al., 2012), abalone mollusks (Zhuang et al., 2015), *Fenneropenaeus chinensis* (Qiu et al., 2014), and *Litopenaeus vannamei* (Petit et al., 2016; Zhang et al., 2017). Numerous researches have pointed out that the potential of haemocyanin activity against bacteria (Lee et al., 2003), viruses (Zhang et al., 2004), fungi (Destoumieux-Garzon et al., 2001), homogeneous erythrocytes (Zhang et al., 2009), and cancer cells (Riggs et al., 2002).

Even if the antiviral activity of haemocyanin has been documented (Zhang et al., 2004), our knowledge about its antibacterial activity was still scarce. In the present study, the antibacterial activity of purified *Pp*-Hc was evaluated against Gram-positive and Gram-negative bacteria, through zone of inhibition assays. *Pp*-Hc showed maximum killing effect against Gram-negative bacteria if compared to Gram-positive ones. The reason behind this seems to be related to the devoid of a thick peptidoglycan layer in Gram-negative bacteria (Ishwarya et al., 2016). Generally, immune related proteins do not show any target site to damage bacterial cells, they simply perturb the bacterial membrane structure due to their affinity towards it. Haemocyanin exhibited affinity towards bacteria resulting in elimination of the microbial pathogen directly from the internal environment of the potential crustacean host. Our results get supported by Sivakamavalli et al. (2015) reporting that the haemocyanin affinity towards bacteria was noticed in *M. japonicus*

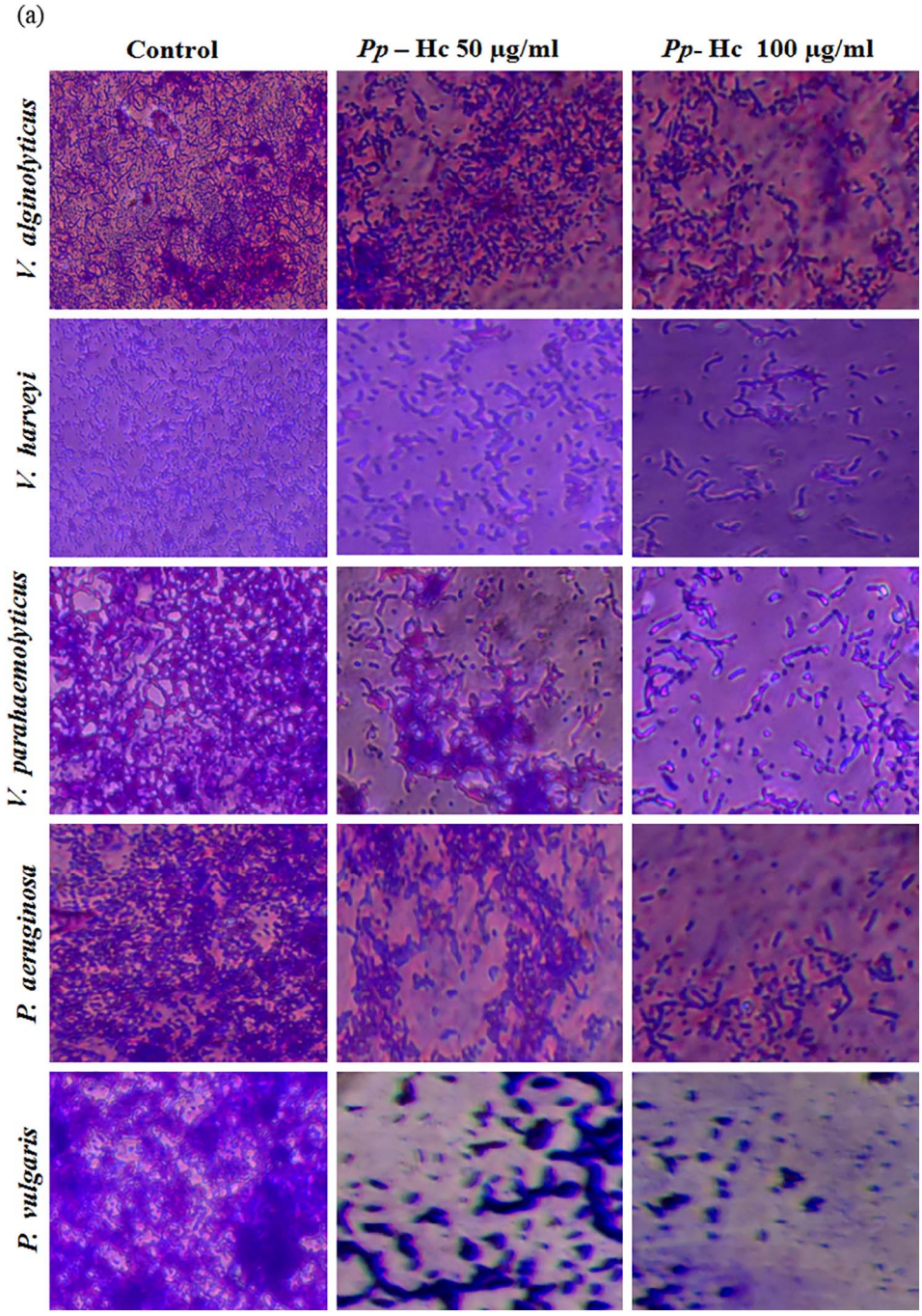

Fig. 9. Effect of haemocyanin purified from the haemolymph of *P. pelagicus* on the biofilm growth of Gram-negative bacteria observed under (a) light and (b) confocal laser scanning microscopy.

haemocyanin, which was able to agglutinate both Gram-positive and Gram-negative bacteria. Due to more prevalence on antibiotic resistance in Gram-negative bacteria, the *Pp*-Hc antibiofilm activity was checked against them, i.e., *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus*, *P. aeruginosa* and *P. vulgaris*. Biofilm growth inhibition and disturbance by *Pp*-Hc at the concentration of 100 µg/ml was visualized by light microscopy and CLSM. For bacterial initial adhesion and biofilm formation, EPS plays vital role acting as a shelter and providing nutrients to biofilm-forming bacteria (Donlan, 2002). In this current attempt, reduction in EPS and elevated CSH led to dispersion of bacterial cells from the polysaccharide matrix. Haemocyanin easily enters inside the EPS matrix, since EPS act as a protective layer to the bacterial colonies.

In conclusion, the haemocyanin anti-bacterial and antibiofilm activity against microbial pathogens widely present in the aquatic environment has been demonstrated in this study, shedding light also on possible mechanisms of action. Haemocyanin is not only involved in transporting oxygen, it acts as a relevant part in the flower crab immune system. Moreover, the antibacterial activity exhibited against *V. harveyi* and *V. parahaemolyticus* is really important in shrimp aquaculture, as these pathogens are being gradually more incriminated in current disease outbreaks resulting in high mortality rates. This work adds useful knowledge for aquaculture concerns dealing with microbial pathogen management (see also Iswarya et al., 2017; Jayanthi et al., 2017). Overall, our results outlined the high potential of purified *Pp*-Hc as a starting material to develop new and effective antimicrobial drugs

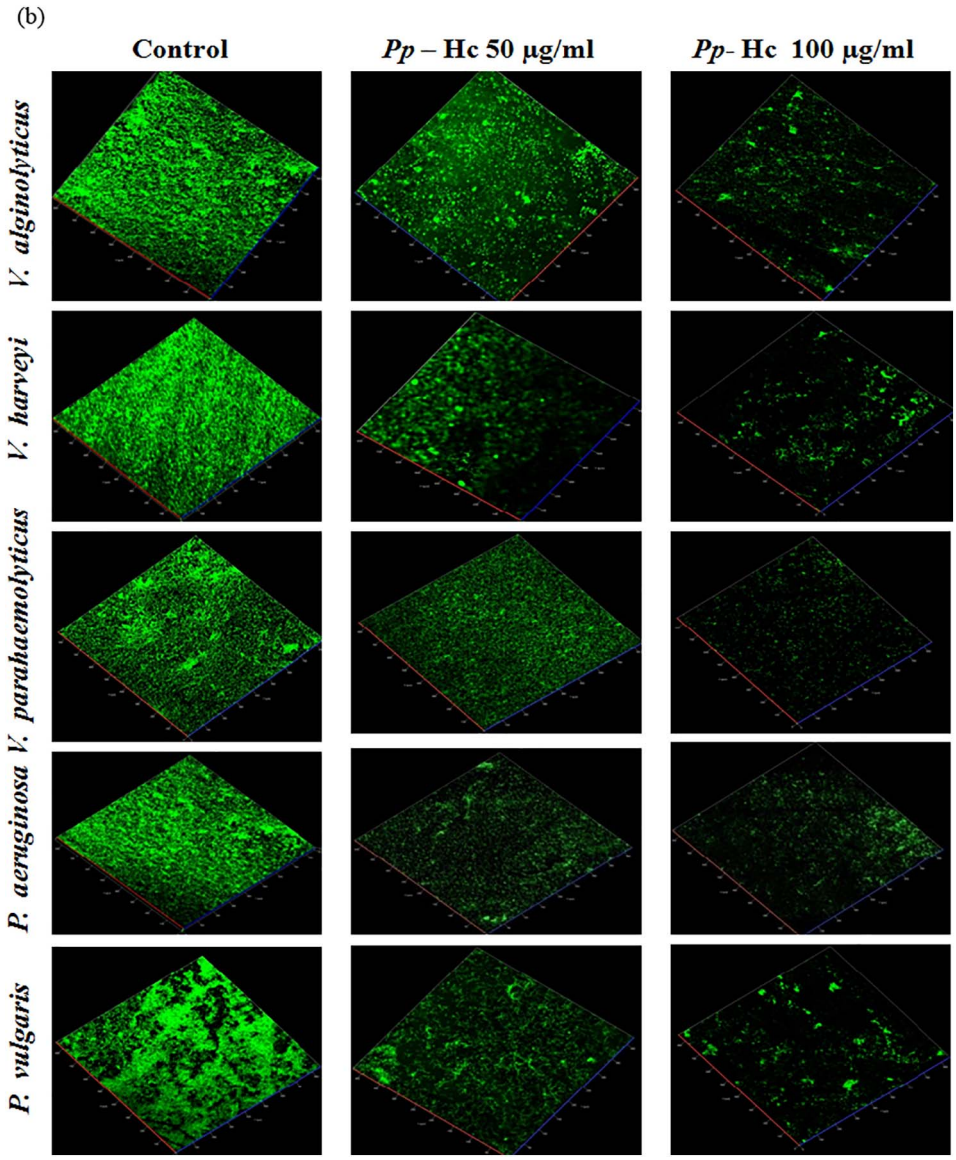

Fig. 9. (*continued*)

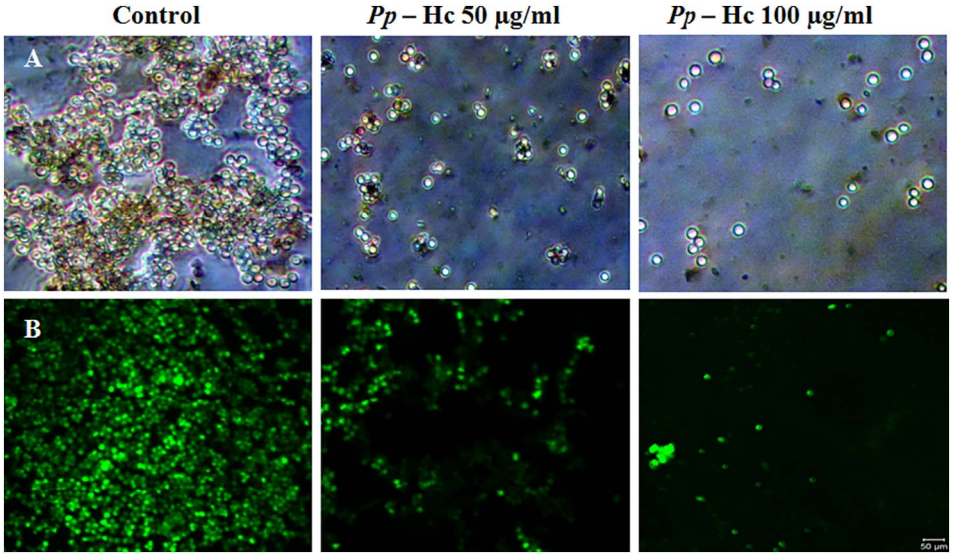

Fig. 10. Effect of haemocyanin purified from the haemolymph of *P. pelagicus* on the biofilm growth of fungal pathogen *Candida albicans* observed under (A) light and (B) confocal laser scanning microscopy.

for aquaculture purposes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2018.02.014.

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