

# Antibiofilm Competency of *Portunus pelagicus* Haemolymph and Identification of its Bioactive Compounds

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## Abstract

The marine atmosphere may be investigated as a rich source for novel drugs. In past decades, a number of marine-derived compounds have been isolated and identified. The current effort was made on haemolymph of blue swimmer crab *Portunus pelagicus* to mark its antimicrobial activity against eight pathogenic bacteria of Gram positive, Gram negative and fungus *Candida albicans*. Agar well diffusion method was put into practice in deliverance of antibacterial and antifungal activity by measuring zone of inhibition. The findings displays, *P. pelagicus* haemolymph at the concentration of 150 µl can effectively act against all of challenged bacteria and fungus. Besides, the antibiofilm property of haemolymph against selected bacteria and fungus *C. albicans* which revealed the potential bactericidal and fungicidal effect at 150 µl concentration of haemolymph. Moreover, the antimicrobial and antibiofilm potent of crab haemolymph was confirmed by growth curve analysis, biofilm growth inhibition and protein leakage assay which collectively conclude the ability of haemolymph in microbial growth inhibition. For the first time, the bioactive compounds were screened from the haemolymph of *P. pelagicus* through Gas chromatography- Mass spectrometry analysis discloses the existence of 15 types of compounds in haemolymph being in charge for antimicrobial activity.

**Keywords:** *Portunus pelagicus*; Haemolymph; Antimicrobial activity; Bioactive compounds

## Introduction

The ocean engages with almost three quarters of the earth's surface and contains an unexpected assortment of life [1]. A diverse array of natural products are seems to be presents in marine ecosystem with abundant of bioactive compounds and have the great potential for the production of pharmacological substances [2]. Part of the biodiversity was inhabited by marine organisms and the marine ecosystem is the greatest source to discover useful therapeutics [3]. Since the numbers of microorganisms which are mastery in quickly changing pattern of resistance to antibiotics have been increased steadfast, the necessities for treating these pathogens demand novel and successful antimicrobial agents. This makes researcher's move forward to the marine wealth due to the exploitation of the terrestrial resources. Bioactive compounds of marine source known to have clinical properties includes antitumor, antiproliferative, anticancer [4,5] antihypersensitive, antimicrotubule, antifouling [6] and antibiotic properties [7-9] and also have cosmeceutical applications [10] At present, only some marine-derived products are currently in the market, quite a few marine natural products are presently in the medical channel, with more undergoing enlargement [11,12]. So far, bioactive compounds for medical utilization were isolated from sponges (37%), coelenterates (21%) and microorganisms (18%), are major sources of biomedical compounds, followed by algae (9%), echinoderms (6%), tunicates (6%), molluscs (2%) bryozoans (1%), etc [13]. However, knowledge about bioactive compounds from arthropods is still insufficient. Being a member of arthropods, crustaceans formed various bioactive substances which posses the antimicrobial activity against pathogenic microbes [14]. In recent decades, the growing number of bacterial strains resistant to conservative antibiotics has evolved as a serious medical problem [15]. Marine pharmacology has been reviewed extensively in the past all over the world as well as in India, but as far as there is an inevitability to review the potential of the marine as foundation for the development of new drugs, considering the advantage of their wealth in nature and bulky invention [16]. Further, chemical compounds secluded

from marine organisms have huge potential as antimicrobials or cytotoxic compounds due to the confidence of marine organisms on antimicrobial composites or cytotoxic molecules as their innate defense devices. Some crustaceans have shown pronounced activities, useful in biomedical area by the action of circulating haemolymph which contains biologically active substances such as complement, lectin [17], clotting factors [18] and antimicrobial peptides [19,20], pattern recognition proteins [21]. Though marine crabs are a source of biologically active products, their importance in biomedical area is largely unexplored. The widespread knowledge in the chemical structure and physiological character of the bioactive substance in the crabs leads to the production of novel drugs with unique action. The crabs are to be prolific source of bioactive compounds but the researchers carried out so far regarding the pharmacological properties are scanty. Hence, an expansive viewing of marine crabs for bioactive compounds is compulsory. In this regard, an endeavour has been made to learn the antibacterial and antifungal activity of the blue swimmer crab *Portunus pelagicus* haemolymph against some selected clinical related pathogens such as Gram positive *Bacillus pumulis*, *Staphylococcus aureus*, *Enterococcus faecalis*; Gram negative *Morganella morganii*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Citrobacter freundii* and fungal pathogen *Candida albicans*. Nevertheless many findings report the

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**Received** July 20, 2016; **Accepted** August 29, 2016; **Published** August 31, 2016

**Citation:** Anjugam M, Iswarya A, Indumathi T, Vaseeharan B, Pachaiappan R, et al. (2016) Antibiofilm Competency of *Portunus pelagicus* Haemolymph and Identification of its Bioactive Compounds. J Aquac Res Development 7: 444. doi: 10.4172/2155-9546.1000444

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antibacterial and antifungal activity of crustacean's haemolymph, to the best of the author's knowledge this is the first attempt to discover the antibiofilm property of crab haemolymph. Formerly, antimicrobial activity of crustacean's haemolymph was reported in *Charybdis lucifera* [22], *Paratellphusa hydrodromous* [23] and *Portunus segnis* [24]. However, there was a lacking to summarize the compounds which are answerable for inhibiting the bacterial growth. In this regard, through GC-MS analysis, bioactive compounds were known from *P. pelagicus* haemolymph.

## Materials and Methods

### Collection of crabs and haemolymph

The healthy crab *P. pelagicus* were collected from the coastal area of Thondi, Ramanathapuram District, Tamil Nadu, India and brought to the laboratory for the collection of haemolymph. Healthy intermoult animals with  $7 \pm 1$  cm width of carapace were maintained in FRP tanks fed twice daily with minced fish meat at 10% of body weight. Healthy crabs of both sexes were used throughout the experimental period, and each crab was subjected to a single bleed. With the help of fine sterile scissors, right chelate leg was gently lacerated in order to get approximately 3 ml of haemolymph from healthy live animal. To void the risk of haemocyte degranulation and coagulation, haemolymph was readily stabilized with anticoagulant solution (Dextrose- 10.25g, Tris sodium citrate-4 g, citric acid - 0.28 g, NaCl- 2.10 g, D.H<sub>2</sub>O-500 ml) and stored at 4°C until use [25].

### Gas chromatography-Mass spectrometry (GC-MS) analysis of *P. pelagicus* haemolymph

As describing by Lawal et al., [26] GC-MS analysis was carried out on a GC-MS (Agilent 7890B GC Connected with 5977A MSD mass spectrometry) comprising an AOC-20i auto-sampler and gas chromatograph interfaced to a mass spectrometer (GC-MS). The instrument is equipped with a VF 5 ms fused silica capillary column of 30 m length, 0.25 mm diameter and 0.25 µm film thickness. The temperatures employed were; column oven temperature 80°C, Injection Temp 250°C at a pressure of 108.0 kPa, with total flow and column flow of 6.20 ml/min and 1.58 ml/min respectively. The linear velocity was 46.3 cm/sec and a purge flow of 3.0 ml/min. The GC program ion source and interface temperature were 200°C and 250°C respectively with solvent cut time of 2.50 min. The MS program starting time was 3.00 min which ended at 30.00 min. with event time of 0.50 sec, scan speed of 1666 µl/sec, scan range 40-800 ul and an injection volume of 1 µl of the *P. pelagicus* haemolymph (split ratio 10:1). The total running time of GC-MS was 30 min. The relative percentage of the compounds was expressed as percentage with peak area normalization.

### Microbial strains

Antimicrobial activity of *P. pelagicus* haemolymph was tested against Gram positive *Bacillus pumulis* (HQ693273), *Staphylococcus aureus* (ATCC 9542), *Enterococcus faecalis* (HQ693279.1), Gram negative *Morganella Morganii* (KC465904), *Proteus vulgaris* (HQ640434), *Pseudomonas aeruginosa* (HQ693272), *Vibrio parahaemolyticus* (KC476545), *Citrobacter freundii* (KC4665905) bacteria and also pathogenic fungi *Candida albicans* (MTCC 7315). Testing organisms were grown in nutrient broth and potato dextrose broth (Hi-Media Laboratories, Mumbai, India) at 37°C for 24 hrs or 48 hrs, subcultured into fresh broth and raised to log phase. By centrifuging at 2000 rpm for 10 min, bacteria and fungus were harvested and washed twice with sterile phosphate buffer saline (PBS) and resuspended in PBS. Taking

absorbance of 0.4 at 570 nm, microbial suspensions get standardized to give 10<sup>9</sup> CFU /ml.

### Antibacterial and antifungal effect of haemolymph from *P. pelagicus*

Employing agar well diffusion method, antibacterial and antifungal potent of *P. pelagicus* haemolymph was assessed against Gram positive, Gram negative bacteria include *B. pumulis*, *S. aureus*, *E. faecalis*, *M. morganii*, *P. vulgaris*, *P. aeruginosa*, *V. parahaemolyticus*, *C. freundii* and also pathogenic fungus *C. albicans*. Overnight cultures of old bacteria and fungi were uniformly spread on the surface of nutrient agar and potato dextrose agar plate using sterile cotton swabs respectively. With the support of sterile cork borer, 7 mm wells were digged and each well were introduced with various concentration of *P. pelagicus* haemolymph (50, 100 and 150 µl) and incubate for 24 hrs and 48 hrs at 37°C for bacteria and fungus respectively. Well devoid of haemolymph was assumed as a control. After incubation, activity was measured in the diameter of millimetre and recorded.

### Growth curve analysis

The impact of haemolymph on the bacterial and fungal growth kinetics was evaluated through growth curve analysis by the method of Maiti et al., [27] with little modifications. In brief, both Gram positive and Gram negative bacteria were cultured in liquid media (nutrient broth and potato dextrose broth) in the presence of haemolymph at three different concentrations (50, 100 and 150 µl). Absence of haemolymph in growing media severed as a control. For every 6 hrs interval, absorbance was measured at 600 nm using UV-visible spectrophotometer (UV-1800; Shimadzu, Japan) and growth curve was plotted.

### Effect of *P. pelagicus* haemolymph on protein leakage from bacterial and fungal cell membranes

Oozing of membrane protein from bacterial and fungal cell wall due to the action of *P. pelagicus* haemolymph was check out through protein leakage assay [28]. Together with diverse concentration of haemolymph (50, 100, 150 µl), microbial cells (10<sup>9</sup> CFU/ml) were incubated in a shaking incubator at 37°C for 6 hrs. Subsequently, incubated samples were centrifuged for 30 min at 300 rpm; the resultant supernatant was treated with reagents of Lowry method. At last, O.D was measured at 595 nm to judge the amount of protein leaked.

### Biofilm growth inhibition

By following the methodology of Sandasi et al. [29] inhibition of biofilm growth was predicted with minor modifications. Prior to the addition of haemolymph, biofilm was allowed to grow in the 96 well plate for 24 hrs. After biofilm growth, haemolymph of different concentration was introduced into the wells rather than control well devoid of haemolymph and allowed to incubate for 24 hrs at 37°C. At the end of incubation, 125 µl of ethanol was added to remove the excess of stain from the wells. Finally 100 µl of destained solution was transferred to fresh plate from the well to take absorbance at 590 nm using ELISA plate reader. Percentage of biofilm inhibition was calculated by following formula.

$$\text{Biofilm inhibition (\%)} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

### Biofilm biomass assessment

Biofilm inhibition of haemolymph was carried out following the

method of Vinoj et al., [30] with slight modifications. By employing staining technique, biofilm biomass in sterile glass pieces was assessed after the incubation of different concentration of haemolymph along with biofilm formed bacteria. After incubation, glass pieces were removed and gently washed with distilled water in order to remove non adherent bacterial cells. The washed glass pieces were air dried, flooded with the stain of acridine orange (0.4%) and crystal violet (0.1%) for confocal laser scanning microscopy (Carl Zeiss LSM 710) and inverted light microscopy (Nikon Eclipse Ti 100X) analysis respectively. Prior to visualize the biofilm inhibition under microscope, glass pieces were gently rinsed with distilled water to remove excess stain and the images were photographed at the magnification of 40X.

## Results

### Gas Chromatography-Mass Spectrometry analysis (GC-MS) of *P. pelagicus* haemolymph

Chromatogram was referred with the database of spectrum of known components stored in GC-MS library for identifying the compounds related to the peak. From the results of GC-MS analysis, approximately 15 peaks analogous to diverse bioactive compounds with various retention times were identified from the haemolymph of *P. pelagicus* (Table 1). Existence and concentration of bioactive compounds in the haemolymph of *P. pelagicus* was directly proportional to height intensity and retention time of each peak (Figure 1). The first peak

| Peak no. | Retention time(RT) | Compound name                      | Nature of compound | Molecular formula(MF)                          | Molecular weight (MW) |
|----------|--------------------|------------------------------------|--------------------|--|-----------------------|
| 1.       | 22.605             | Phenol 2,4-bis 1,1 -dimethyl ethyl | Phenol             | C <sub>14</sub> H <sub>22</sub> O              | 206                   |
| 2.       | 27.085             | Hexadecane,2,6 11,5-tetramethyl    | Fatty acid         | C <sub>20</sub> H <sub>42</sub>                | 282.32                |
| 3.       | 29.135             | 3-Hexadecanol                      | Alcohol            | C <sub>16</sub> H <sub>34</sub> O              | 242.26                |
| 4.       | 29.581             | Isopropyl myristate                | Ester              | C <sub>17</sub> H <sub>34</sub> O <sub>2</sub> | 270.2                 |
| 5.       | 30.487             | 1,2-Benzenedicarboxylic acid       | phthalate          | C <sub>16</sub> H <sub>12</sub> O <sub>4</sub> | 278.15                |
| 6.       | 31.423             | Bis(2-methyl propyl) ester         | phthalate          | C <sub>16</sub> H <sub>22</sub> O <sub>4</sub> | 278.15                |
| 7.       | 31.832             | Phthalic acid                      | phthalate          | C <sub>17</sub> H <sub>24</sub> O <sub>4</sub> | 292.16                |
| 8.       | 32.040             | Hexadecanoic acid                  | Fatty acid         | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub> | 280.46                |
| 9.       | 32.366             | Dibutylphthalate                   | phthalate          | C <sub>18</sub> H <sub>28</sub> O <sub>4</sub> | 312.42                |
| 10.      | 32.775             | Phthalic acid                      | phthalate          | C <sub>17</sub> H <sub>24</sub> O <sub>4</sub> | 292.16                |
| 11.      | 33.191             | Bis(2-pentyl ester)                | phthalate          | C <sub>18</sub> H <sub>26</sub> O <sub>4</sub> | 306.18                |
| 12.      | 33.406             | 1,2 benzenedicarboxylic acid       | phthalate          | C <sub>20</sub> H <sub>30</sub> O <sub>4</sub> | 334.21                |
| 13.      | 33.785             | Di(2-methyl butyl ester)           | Ester              | C <sub>18</sub> H <sub>30</sub> O <sub>4</sub> | 306.18                |
| 14.      | 34.224             | Butyl 3-methyl butyl ester         | Ester              | C <sub>17</sub> H <sub>24</sub> O <sub>4</sub> | 292.16                |
| 15.      | 35.435             | Cyclohexylmethylbutyl ester        | Ester              | C <sub>19</sub> H <sub>26</sub> O <sub>4</sub> | 318.18                |

Table 1: Bioactive compounds identified in the haemolymph of *P. pelagicus* through GC-MS analysis.

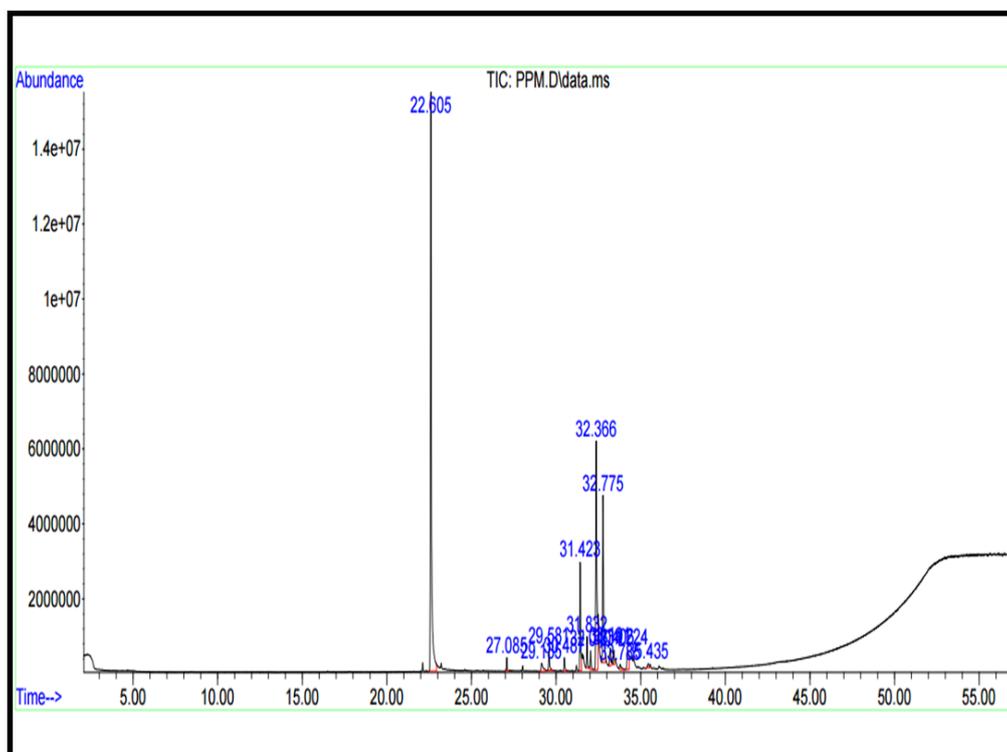
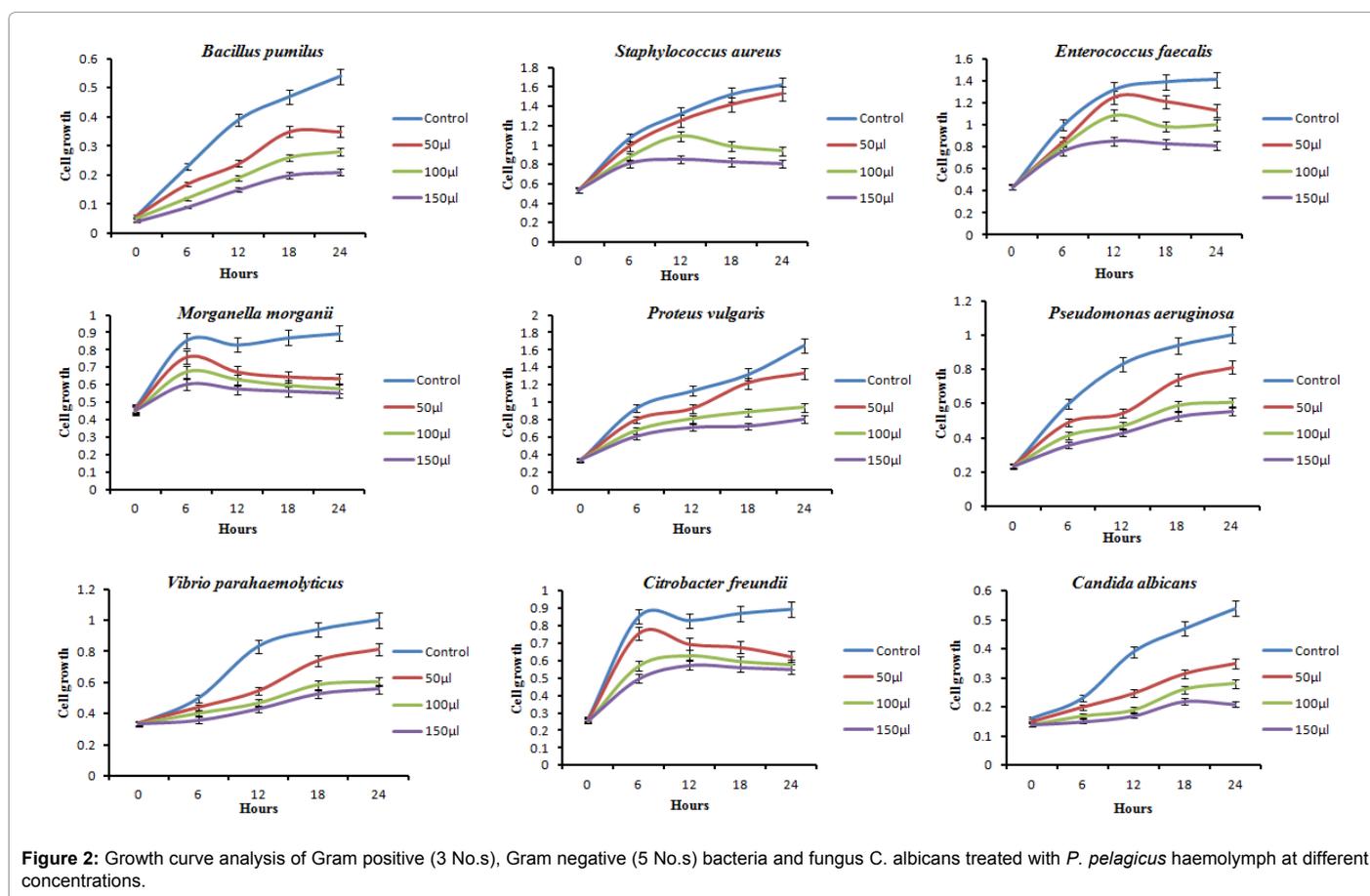


Figure 1: Presence of various bioactive compounds in *P. pelagicus* haemolymph were confirmed by Gas chromatography - Mass spectrometry spectrum with different retention time.

| S.No | Tested organisms                | Accession number | Control | 50µl | 100 µl | 150 µl |
|------|---------------------------------|------------------|---------|------|--------|--------|
| 1.   | <i>Bacillus pumilus</i>         | HQ693273         | -       | +    | ++     | +++    |
| 2.   | <i>Staphylococcus aureus</i>    | ATCC 9542        | -       | +    | ++     | +++    |
| 3.   | <i>Enterococcus faecalis</i>    | HQ693279.1       | -       | +    | ++     | +++    |
| 4.   | <i>Morganella morganii</i>      | KC465904         | -       | +    | ++     | +++    |
| 5.   | <i>Proteus vulgaris</i>         | HQ640434         | -       | +    | ++     | +++    |
| 6.   | <i>Pseudomonas aeruginosa</i>   | HQ693272         | -       | +    | ++     | +++    |
| 7.   | <i>Vibrio para-haemolyticus</i> | KC476545         | -       | +    | ++     | +++    |
| 8.   | <i>Citrobacter freundii</i>     | KC4665905        | -       | +    | ++     | +++    |
| 9.   | <i>Candida albicans</i>         | MTCC 7315        | -       | +    | ++     | +++    |

(+) susceptibility inhibition zone lesser than 5 mm  
 (++) susceptibility inhibition zone lesser than 7 mm  
 (+++) susceptibility inhibition zone greater than 7 mm  
 (-) no inhibition

Table 2: Antibacterial activity of *P. pelagicus* haemolymph against Gram positive (3 Nos), Gram negative (5 Nos) bacteria and fungus *C. albicans*.

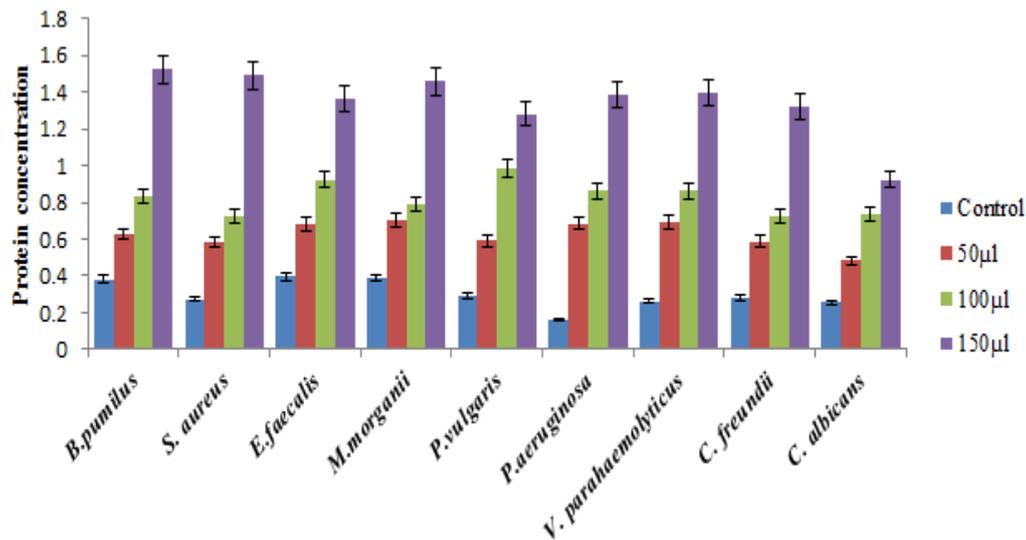


corresponding to phenol-2, 4-bis 1, 1- dimethylethyl (206 kDa) with the retention time of 22.605 min displays high concentration whereas cyclohexylmethyl butyl esters (318.18 kDa) with the retention time of 35.435 min shows low concentration abundance in the haemolymph. Biocompounds in *P. pelagicus* haemolymph express different nature of compounds include phenol, fatty acids, alcohol, phthalate and esters. Among that, substance express phthalate nature was more in number. Thus, each compound manifest diverse nature may be actively involved in silencing the life of microbes.

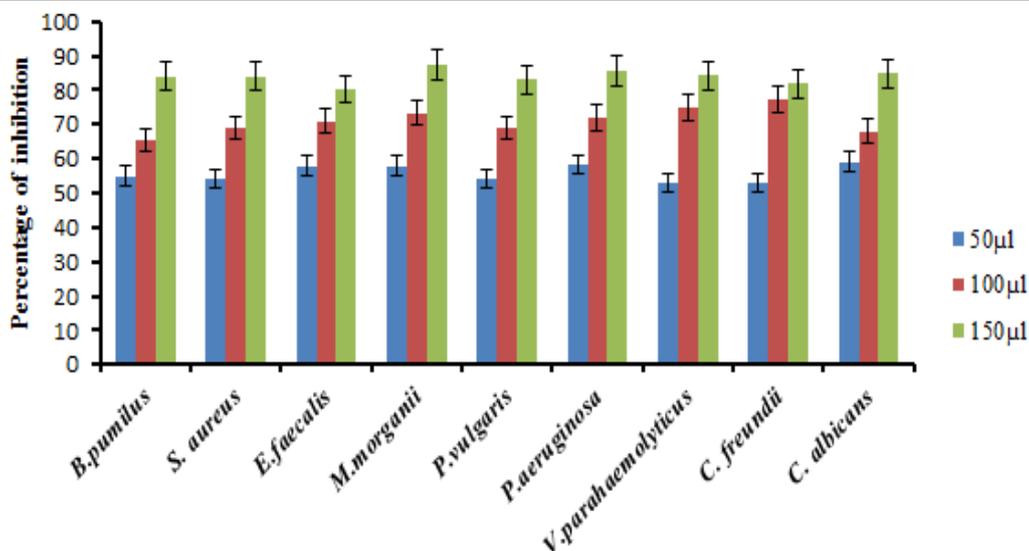
### Antibacterial and antifungal activity of haemolymph

Sensitivity of Gram positive, Gram negative bacteria and fungus *C. albicans* to crab haemolymph was evaluated by agar well diffusion

method. Gram positive and Gram negative bacteria showed high sensitivity when exposed to higher concentration of haemolymph. Haemolymph of *P. pelagicus* was observed to be an effective agent in preventing the growth of Gram positive, Gram negative bacteria and fungus *C. albicans*. After the addition of *P. pelagicus* haemolymph, the intensification of Gram positive (3 No.s) and Gram negative (5Nos) was reduced when compared to the untreated (Table 2). Zone of inhibition was increased at the concentration of 100 and 150 µg/ml and there was no reticence occurs at control. Agar well diffusion assay attested that the concentration of 100 and 150 µg/mL of crab haemolymph showed effective inhibitory activity against tested microbes with different range of zone of inhibition. Increase the concentration of *P. pelagicus* haemolymph showed the increased zone size against both Gram



**Figure 3:** Protein leakage concentration due to bacterial membrane damaging action of *P. pelagicus* haemolymph against Gram positive (3 No.s), Gram negative (5 No.s) bacteria and fungus.



**Figure 4:** The effect of *P. pelagicus* haemolymph on the growth of microbial biofilm expressed as percentage of inhibition.

positive and Gram negative bacteria. Besides, *P. pelagicus* haemolymph also exhibits antifungal activity against the fungal pathogen *C. albicans*.

### Growth curve analysis

The slant of bacterial growth curve was declined in the presence of *P. pelagicus* haemolymph at higher concentration. This explains that, the growth of bacteria was indirectly proportional to haemolymph concentration (Figure 2). The results clearly explain that, at low concentration of haemolymph, the growth of bacteria was delayed and at higher concentration growth was inhibited. Therefore, it can be concluded that haemolymph may acts as bacteriostatic at low concentration and bactericidal at high concentration. It was noticed that, high concentration of haemolymph has the ability to hold the bacterial growth and wipe out it. It was experiential that at lower initial OD, the haemolymph concentration necessary to completely inhibit bacterial growth was also low.

### Effect of *P. pelagicus* haemolymph on protein leakage from bacterial and fungal cell membranes

It was found that haemolymph happen to reason for protein leakage by increasing the membrane permeability of Gram positive, Gram negative bacteria and fungus (Figure 3). At first, protein leakage from the membranes of bacteria and fungus *C. albicans* treated with haemolymph was almost the same as that from cells in the control group. At 6 hrs after incubation, protein leakage from cells treated with haemolymph considerably increased; however, there was no change in the amount of protein leakage from cells in the control group indicating that haemolymph can increase the membrane permeability leads to the release of internal materials. Notably, higher amounts of proteins leaked through the Gram negative membranes compared to those through the Gram positive membranes because of thick peptidoglycan layer.

### Biofilm growth inhibition

Growth of biofilm inhibition with spectrophotometric assay clearly read out the potential of haemolymph on biofilm inhibition. The result demonstrates, all the tested concentration of haemolymph directly affects the growth of preformed biofilm of all challenged bacteria. Also biofilm growth inhibition conveys, at very low concentration of haemolymph (50  $\mu$ l) noticeable result was observed and thereby effect of haemolymph was more pronounced on biofilm when the concentration get elevated (Figure 4). Take as a whole, haemolymph of a *P. pelagicus* showed more than 75% potent in inhibit the growth of bacteria.

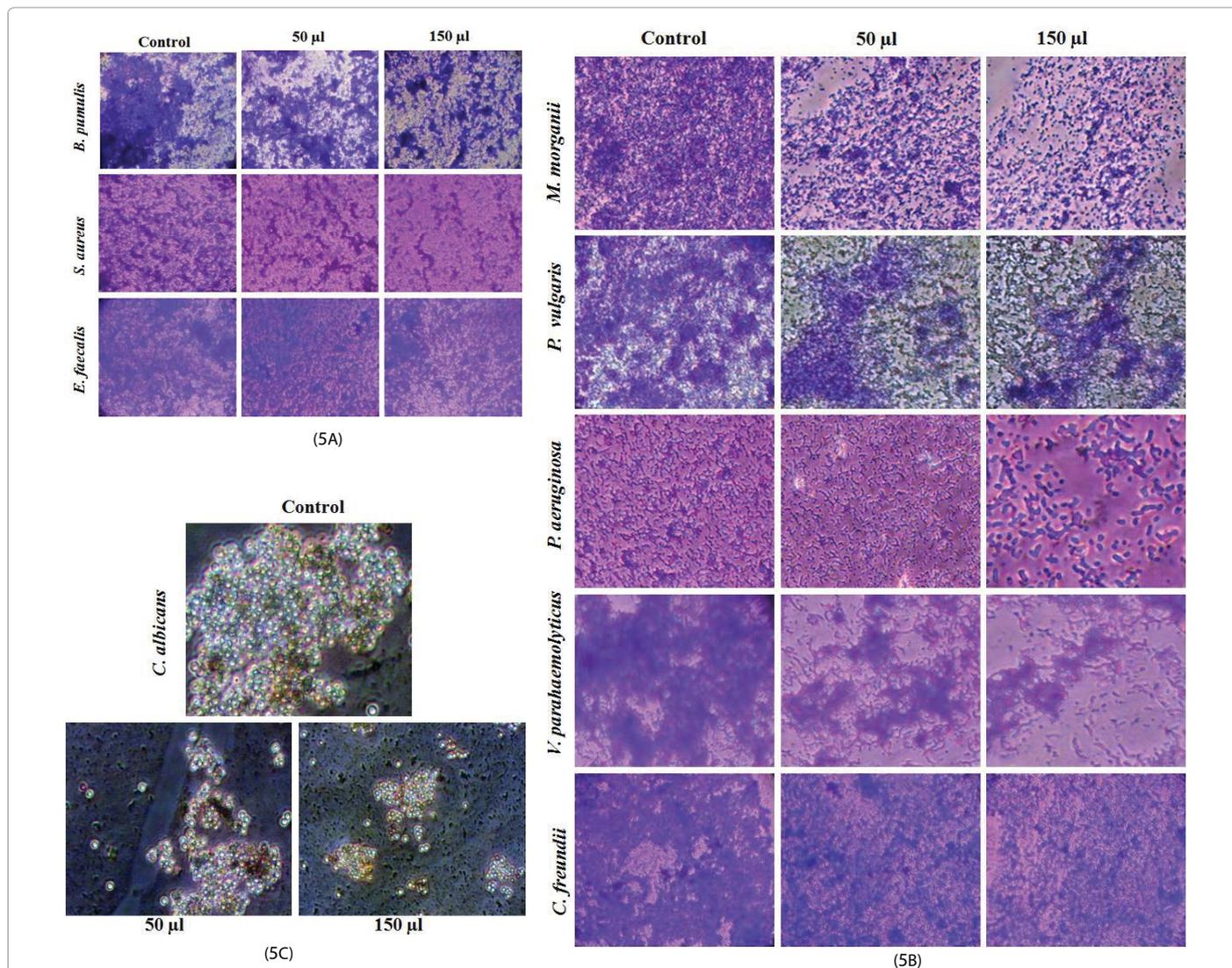
### Antibiofilm potential of *P. pelagicus* haemolymph

Confocal laser scanning microscopy and light microscopy captures reveals *P. pelagicus* haemolymph effectively disrupt the biofilm construction by reducing the thickness of the exopolysaccharide found on the outer surface of both Gram positive and Gram negative bacteria and also fungal pathogen *C. albicans* (Figures 5 and 6). The impact on

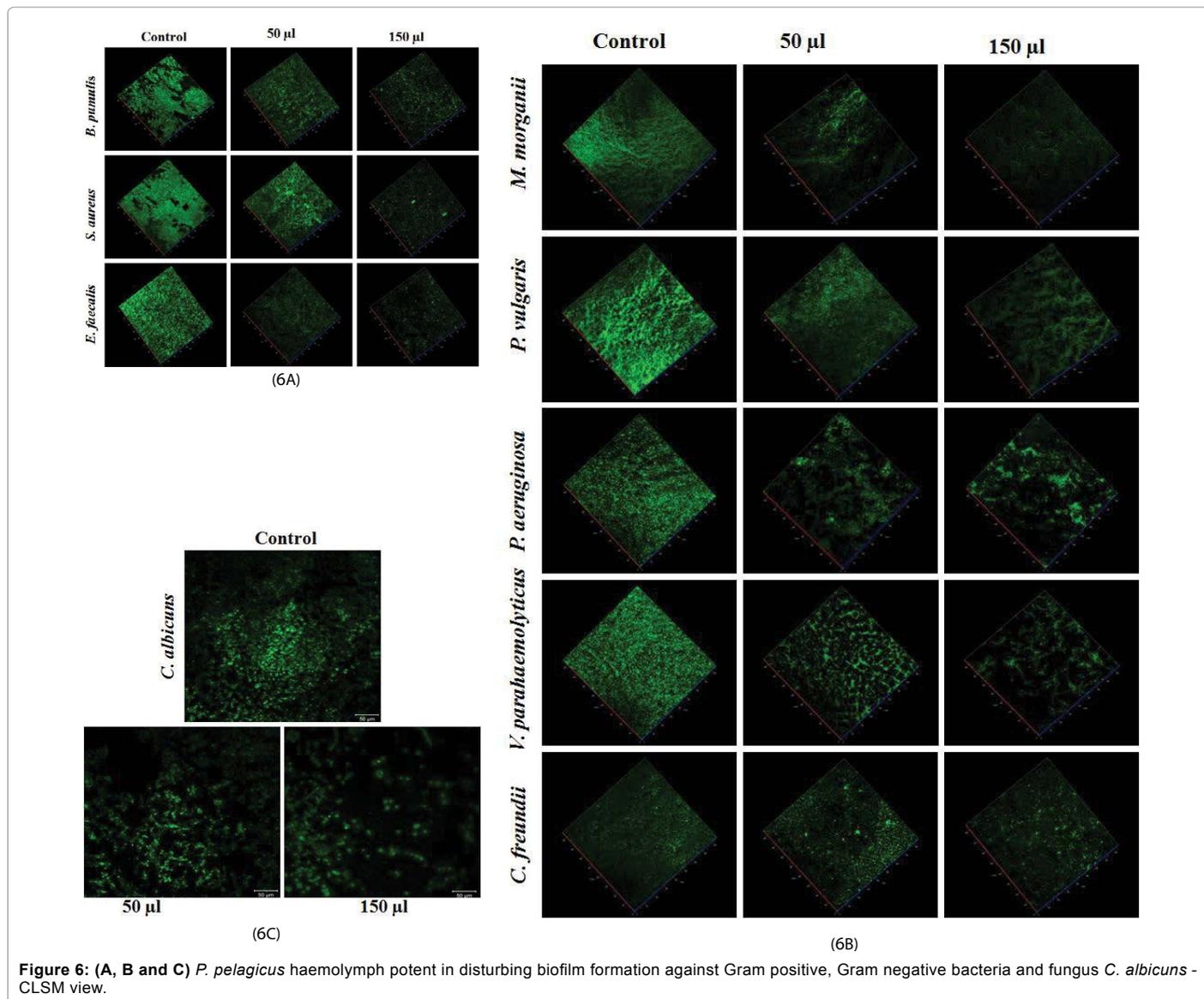
biofilm inhibition was demonstrated with various concentrations of haemolymph have potential inhibitory effect on biofilm formation. At the concentration of 150  $\mu$ l haemolymph acts well upon the bacterial and fungal biofilms and there was a proportional decrease in thickness of biofilm with increased concentration.

### Discussion

Haemolymph of invertebrates pay great attention to perceive novel bioactive compounds for pharmacological utilization. Among invertebrates, crustaceans include crabs acts as a store house of antimicrobial substance with wide range of biomedical properties. On this basis, this current study was undertaken to let out the antimicrobial property of blue swimmer crab *P. pelagicus* haemolymph against various bacterial strains of both Gram positive and Gram negative bacteria comprise *B. pumulis*, *S. aureus*, *E. faecalis*, *M. morgani*, *P. vulgaris*, *P. aeruginosa*, *V. parahaemolyticus*, *C. freundii* and fungal strain *C. albicans*. Antimicrobial activity of crab haemolymph has been reported in the haemolymph of the blue crab *Callinectes sapidus* [31], mud crab *Scylla serrata* [32,33], Ghost crab



**Figure 5:** (A and B) Disruption of biofilm formation of Gram positive and Gram negative bacteria by *P. pelagicus* haemolymph was visualized under light microscopy with the stain of crystal violet; (C) Antibiofilm property of haemolymph on fungus *C. albicans*.



*Ocyptode macrocera* [34] and Littoral crab *Carcinus maenas* [35], As yet, antimicrobial activity of haemolymph get studied, the bioactive compounds behind antimicrobial action is not elucidated. In this study, we report the antimicrobial potent of *P. pelagicus* haemolymph and existence of bioactive compounds answerable for antimicrobial activity. Through GC-MS analysis, haemolymph show 15 important bioactive compounds with varying nature at different retention times notable to have antimicrobial property. Among that, phenol-2, 4-bis (1, 1 dimethylethyl) concentration was abundantly accounted and known to force antibiofilm activity. Studies investigated phenol-2, 4-bis (1, 1 dimethylethyl) from marine origin directly inhibit biofilm forming bacteria *Serratia marcescens* [36]. In this attempt, phthalate nature compounds occupancy was more number in haemolymph of *P. pelagicus*; thereby its contribution in antibacterial action was higher than other compounds [37,38]. In addition to the above mentioned bioactive compounds, esters, fatty acids, alcohol were actively involved in microbicidal job together with or without other bioactive groups. Thus, haemolymph of *P. pelagicus* certified as rich source of defence molecule against pathogens. In this study, haemolymph of *P. pelagicus*

reveals its potent lethal action against 8 selective Gram positive and Gram negative pathogenic bacteria also includes infective fungus *C. albicans* by killing effect measured by zone of inhibition. The mortal action of haemolymph may achieve due to interrupting with bacterial membrane to turn over intracellular mechanisms. Parallel results were accounted in the haemolymph of some brachyuran crab against clinical pathogen [18,39,40]. Interfering and inhibition of bacterial growth was important feature for good bioactive substance. In this way, growth kinetics was taking up with bacterial and fungus cultures treated with haemolymph of *P. pelagicus* result in the inhibition of microbial growth. This is attained by interference of *P. pelagicus* haemolymph on log phase and thereby reduces the growth pattern leads death of bacteria. By look over the result of GC-MS chromatogram, fatty acid in the *P. pelagicus* haemolymph go in for the bacterial growth suppression action via creating unfavourable condition to the challenging bacteria [41]. Biofilm- a mass of bacterial colony sheltered by polysaccharide matrix is another serious concern under bacterial infection. Thus far, antimicrobial activity of haemolymph have been reported, antibiofilm activity of haemolymph is still not announced. Output of our study

reports antibiofilm activity of *P. pelagicus* haemolymph on biofilm construction. In our previous studies, biofilm architecture of different pathogenic bacteria was inhibited by microbial based silver nanoparticles [42], protein based gold nanoparticles [30], pattern recognition protein  $\beta$ -GBP [21] and essential oil coated gold nanoparticles [43]. This is the first attempt to find out the antibiofilm potential of crab haemolymph. In biofilm, the shelter exopolysaccharide provide great protection to bacterial colonies by preventing the penetration of any antibiotic drugs [44]. However, *P. pelagicus* haemolymph show exception to the above fact, because it penetrates and disturb the polysaccharide matrix composed by microbial population. This is supported by antibiofilm activity where haemolymph perturb the pool of biofilm forming bacteria, result in collapsing biofilm architecture. CLSM and light microscopic view clearly shows *P. pelagicus* haemolymph upset bacterial cells so that bacterial cells get dispersed from their colony or substratum. The disturbance of *P. pelagicus* haemolymph was accomplished from least concentration (50  $\mu$ l) and increased with high concentration (150  $\mu$ l). In above said action, fatty acid and phenol in *P. pelagicus* haemolymph are highly engaged in producing repulsive action between microbial cells and substratum. Microbial cells from their substratum and so microbial cells get dispersed and biofilm formation was stopped. Also alcoholic compounds in the haemolymph of *P. pelagicus* show ability to disturb bacterial cell membrane. This may cause acidic nature to cytoplasmic environment leads to denaturation of enzymes and other functions get collapsed to kill microbes. Our suggestion correlate with the view of Ingram, 1990 [45] clearly report the killing mechanism of alcohol. By employing Lowry method, protein spill from the bacterial cell membrane was quantified indicating bacterial cell damage due to the action of *P. pelagicus* haemolymph. This clearly demonstrate haemolymph of *P. pelagicus* penetrate into the bacterial cells by increasing bacterial cell membrane flexibility. As a consequence, the present report uncovers the antimicrobial property of *P. pelagicus* haemolymph. Thus, *P. pelagicus* haemolymph acts as a good antimicrobial agent satisfy the needs of pharmaceutical industries in generating novel drugs against antibiotic resistant bacteria. Further studies will be focused towards the elucidation of potential bioactive compounds and their inhibition mechanism on microbial growth.

## Conclusion

In conclusion, haemolymph of *P. pelagicus* displays its potent as antimicrobial agent against some pathogenic bacteria and fungus. As a circulating fluid of crustaceans, haemolymph not only protect the animal, but also bioactive substance rich haemolymph acts as a good antimicrobial agent to mankind. This may attract the attention of pharmaceutical industry to introducing novel drugs with natural activities against multi drug resistant pathogenic microbes.

## Acknowledgement

This work was supported by the Department of Biotechnology (DBT), New Delhi, India, under the Project grants code BT/PR7903/AA/3/638/2013.

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