Screening of hemolymph of marine crab *Tumidodromia dromia* for its antibacterial and hemagglutination property

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ABSTRACT

Marine crabs, which survive in a stressful environment, have developed modalities to overcome stress and pathogens by the production of innate immune molecules. These humoral factors have been tapped as an alternative source of natural drugs. This study is aimed to assess the antimicrobial properties and screen for the presence of agglutinins in the hemolymph of the marine crab Tumidodromia dromia through disc diffusion method and hemagglutination assay. In-vitro antibacterial activity of the hemolymph against Bacillus cereus, Bacillus subtilis, Klebsiella pneumonia, Enterobacter aerogenes, Proteus mirabilis and Escherichia coli was carried out following disc diffusion method. The highest antibacterial activity of the crab's hemolymph over Bacillus cereus, Enterobacter aerogenes and Bacillus subtilis was observed in a dose response manner. Since the hemolymph exhibited antimicrobial properties, it was hypothesized that agglutinins could be a factor influencing antimicrobial activity. To test this hypothesis, the hemagglutination activity of the hemolymph was carried out with the erythrocyte derived from cow, goat, buffalo, pig, mice, guinea pig, rabbit, rat and chick. The agglutination reaction was seen maximum with erythrocytes derived from buffalo, mice and guinea pig. The result of the study revealed that the hemolymph of Tumidodromia dromia has hemagglutination properties. Purified form of antimicrobial molecule and hemagglutinin from the hemolymph of the crab can be used as natural drugs against microbes and cancer.

Keywords: *Antimicrobial activity, Crab lectin, Hemagglutination, Hemolymph.*

1. Introduction

Microorganisms have developed resistance against drugs and it is primarily due to misuse and overuse of drugs. Antimicrobials are designed to fight against the pathogenic microbes but these microbes restrict access to the antibiotics by preventing their entry through the cell wall by either pumping mechanism or degrading with the help of enzymes and proteins [1]. Isolation of antimicrobial substances from natural substances has found to be an alternative option [2]. According to recent studies the solution lies within the marine environment and the developing countries have now turned their spotlights towards marine life. Ocean is a host of diverse organisms and is constantly under the threat of pathogenic microbes and other stress

factors. To combat the stress, various peptides, alkaloids, polyketides and terpenes are produced by the microbes which in turn have been found to exert antimicrobial activity [3]. Invertebrates and vertebrate animals have two kinds of defence against microorganisms: cellular and humoral mechanisms. The crustacean humoral immunity is characterized by antimicrobial factors like clotting factors, lipopolysaccharides, peptides, complements and lectins which are found in the circulating hemolymph. The marine crab which can be used as a source of natural antibiotics has a hard cuticle as the first defence mechanism. Complex interactions of innate humoral and cellular immune response are observed in tissues and hemocoel, which leads to elimination of pathogens. Clear understanding of the biological activity can help immensely in the formalization of new drugs with specific action [4]. The crab hemolymph is a source of antimicrobial molecules such as cathelicidins, protegrins, lectins etc. [5]. Lectin provides a role of blockade of invasion, infection, inhibition of growth, microbial cell adhesion and migration.

Antimicrobial properties of hemolymph of crab has been documented from marine crabs *Charybdis lucifera* [6, 7], *Carcinus maenas* [8, 9], *Calinectes sapidus* [10, 11], *Liagore rubromaculata*[12], *Ocypode macrocera* [13] and *Atergatis integerrimus* [14]. Hemagglutination has been observed in crabs *Scylla serrata* [15], *Atergatis integerrimus* [16], *Travancoriana charu* [17], *Grapsus tenuicrustatus* [18]. Since marine crabs have been found to be a rich source of biomolecules that can specifically target clinical pathogens, the present investigation was carried out to screen for the antimicrobial property and to find out the presence of agglutinins which could be a potential molecule to target microbes. The hemolymph of the marine crab *Tumidromia dromia*, was selected and the experiment was carried out with the following objectives.

- To evaluate in vitro antibacterial activities against *Escherichia coli, Proteus mirabilis, Klebsiella pneumonia, Enterobacter aerogenes, Streptococcus pyogenes, Bacillus subtilis* and *Bacillus cereus*.
- To screen for the presence of agglutinins in the hemolymph.

2. Materials and Methods

Collection of Hemolymph

The crab *Tumidromia dromia*, were collected from the coastal area of Kela Manakudi, Kanniyakumari, Tamil Nadu, India (Latitude: 8.1161°N, Longitude: 77.4883°E). The hemolymph was collected by cutting the third walking leg of the crab in a centrifuge tube. The crab was then safely let back into the sea.

Bacterial Strains

Gram positive bacteria such as *Bacillus subtilis* (MTCC 5981), *Bacillus cereus* (MTCC 430); Gram negative bacteria such as *Klebsiella pneumonia* (MTCC 530), *Enterobacter aerogenes* (MTCC 111), *Proteus mirabilis* (MTCC 1429), *Escherichia coli* (MTCC 443) were collected from Scudder labs, Nagercoil and maintained at 4°C.

Antibacterial Assay

In vitro antibacterial assay was carried out by disc diffusion technique [20]. Whatmann No 1 filter paper disc with 6 mm diameter was impregnated with different concentrations of hemolymph (25 µl, 50 µl, 75 µl, 100 µl). The discs along with positive control (Standard antibiotic disc) were kept at the centre of Muller Hinton Agar (MHA) plates seeded with bacterial culture. After incubation at room temperature for 24 hours, antibacterial activity expressed in terms of diameter (mm) of zone of inhibition was calibrated and recorded.

Collection of Erythrocytes

Cow, goat, buffalo, pig and chick blood samples were collected from slaughter houses and blood of other animals were collected from veterinary hospitals by ear (rabbit) and heart puncture (rat, guinea pig, mice). Blood samples were collected directly in sterile modified Alseivier's medium pH 6.1 (30 mM sodium citrate, 77 mM sodium chloride, 114 mM glucose, 100 mg neomycin sulphate and 330 mg chloramphenicol). Blood samples were suspended and washed three times with ten volumes of Tris-buffered saline TBS, pH 7.5 (Tris-HCl 50 mM, NaCl 100 mM, $CaCl_2$ 10 mM) and resuspended in the same as 1.5% suspension.

HA Assay

Hemagglutination assay was performed in 96 well, 'U' bottomed microtiter plates (Tarson, India) as described by Ravindranath and Paulson [19]. The hemolymph (25 µl) was serially diluted with TBS (25 µl, pH 7.5) and mixed with 25 µl of 1.5% erythrocyte suspension and incubated for 1 hour at room temperature (30±2°C). HA titre was reported as the reciprocal of the highest dilution of hemolymph giving complete agglutination after 1 hour.

3. Results

Antibacterial Property

Bacterial strains *Bacillus cereus, Bacillus subtilis, Klebsiella pneumonia, Enterobacter aerogenes, Proteus mirabilis* and *Escherichia coli* were screened for antibacterial activity against hemolymph. Different concentrations of hemolymph (25 µl, 50 µl, 75 µl and 100 µl) were tested for all the strains and amikacin was used as control. The bacterial strains were sensitive to the hemolymph with an inhibitory zone ranging from 9 mm to 16 mm. The results

showed that the pathogens were sensitive to the hemolymph in a dose dependent manner (Fig. 1 & plate 1).

Fig 1. The antibacterial activity of crab hemolymph

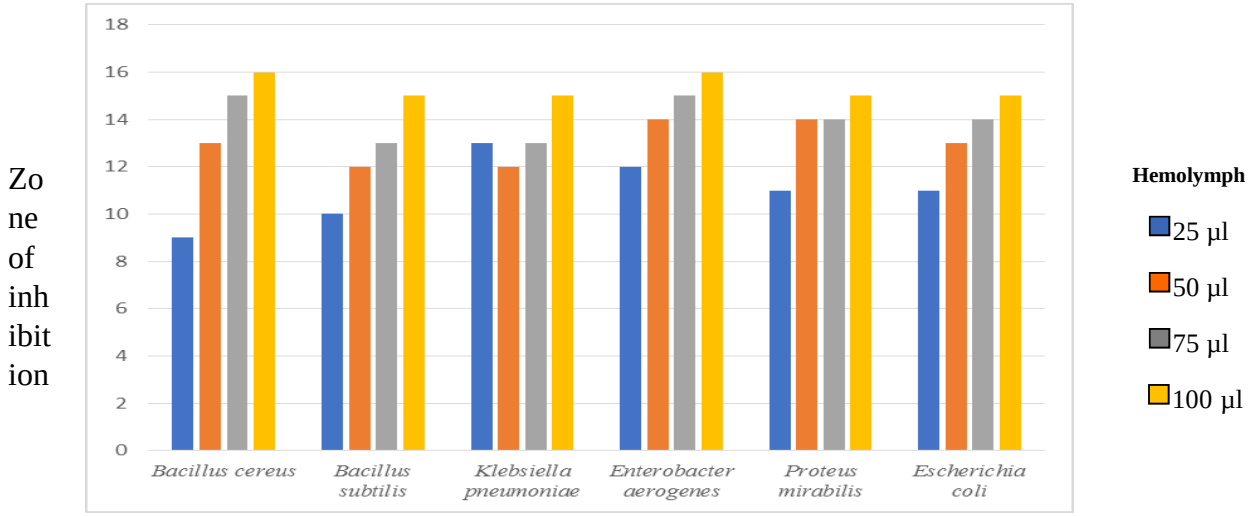


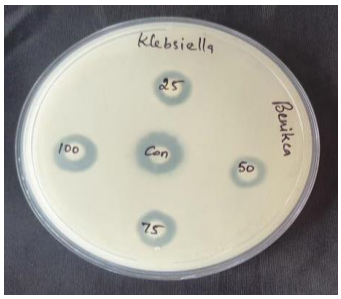
Plate 1. Antibacterial activity of hemolymph against *Bacterial strains*

Bacillus cereus

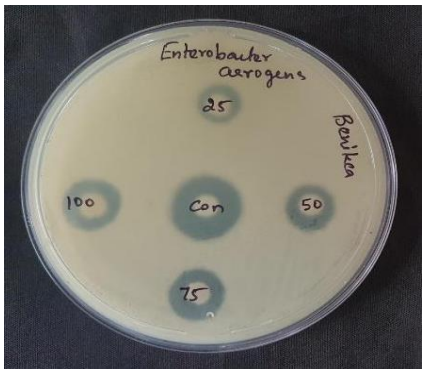
Bacillus subtilis

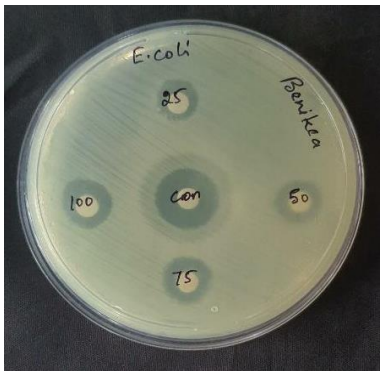
Proteus mirabilis

Klebsiella pneumonia



Enterobacter aerogens *Escherichia coli*





Hemagglutination Property

In order to know whether agglutinins could have contributed to antimicrobial activity HA assay was carried out. Hemagglutination studies revealed the presence of agglutinins as evident by its capacity to agglutinate mammalian erythrocytes. Buffalo, guinea pig and mice erythrocytes showed considerable hemagglutination with the crab's hemolymph (Table 1) when compared to other erythrocytes.

Table 1. Hemagglutinin activity of the hemolymph of the marine crab, *Tumidromia dromia*

Erythrocytes	Cow	Goat	Buffalo	Pig	Mice	Guinea pig	Rabbit	Rat	Chick
HA Titer	2	16	64-128	0	256	64	2	0	2

4. Discussion

Nature always has, still and will serve as the best source of medicines and antimicrobials. With the rise in marine and arthropodan research, effective bioactive compounds are isolated [21] and applied for diagnosis and treatment of diseases. Hence, a marine crab *Tumidodromia dromia* was selected and the hemolymph was collected and tested for antimicrobial activity. The activity was noted for Gram positive, as well as Gram negative bacteria. In the present study, a slight increase in activity was seen against Gram positive *Bacillus cereus* and *Bacillus subtilis;* and also, Gram negative *Klebsiella pneumonia, Enterobacter aerogenes, Proteus mirabilis* and *Escherichia coli*. Antimicrobial activity has been documented from the hemolymph of *P. pelagicus* against Gram-positive *Bacillus pumilus, S. aureus, and E. faecalis*, and Gram-negative *Morganella morganii, P. vulgaris, and P. aeruginosa*, as well as the fungus *Candida albicans* at 150 µg/mL [22]. The density charge,

structure of lipopolysaccharides and lipid composition of the cytoplasmic membrane in Gram negative and Gram-positive bacteria might be the cause [23]. The crude hemolymph of *Carinoscorpius rotundicauda* has a disruptive effect on the peptidoglycan layer of Gram-positive bacteria which destructs the defence mechanism of bacteria [24].

Maximum zone of inhibition of 16 mm and 14.7 mm has been reported against *Enterobacter sp*. and *S. entrica* from male hemolymph and protein precipitated from hemolymph respectively of *Portunus segnis* [25]. The report of Sundaramurthy et al [26] revealed that the hemolymph of the male crab *Uca triangularis* shows an inclined level of antibacterial peptide, than that of female hemolymph. Hemagglutinin assay showed that the hemolymph has affinity towards the erythrocytes tested at varied capacities with HA titer ranging from 2 to 256. The agglutination against buffalo erythrocytes is due to the presence of the receptor component NeuGc, a sialic acid on the glycocalyx of buffalo erythrocytes [27, 16]. The purified antimicrobial peptide and lectin may have greater potential towards pharma chemicals [28]. Since the presence of agglutinins is observed it may be speculated that lectin could be a factor complementing the antimicrobial activity. Thus, the present study paves a way to further isolate the lectin and assess its antimicrobial potential on clinical pathogens.

5. Conclusion

The hemolymph of the marine crab *Tumidromia dromia* has shown antibacterial activity against Gram positive bacteria such as *Bacillus cereus, Enterobacter aerogenes* and *Bacillus subtilis*. Hemagglutination assay showed the presence of agglutinin, which agglutinated buffalo, mice and guinea pig erythrocytes. Upon purification this can lead to a new discovery of antibacterial drugs.

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