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Original article

Identification of a novel antibacterial protein from hemolymph of freshwater zooplankton *Mesocyclops leuckarti*

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ABSTRACT

Bacterial infections are the most important problem of health care worldwide. The hemolymph antibacterial proteins of *Mesocyclops leuckarti* was isolated for the first time and its antibacterial efficacy was evaluated against four different human pathogenic microbes viz., *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Shigella flexneri*. The antibacterial potential of the antimicrobial proteins of hemolymph samples from plankton cultured in water enriched with Cow Urine Distillate (CUD) was compared with normal ones. The results indicated that the hemolymph proteins were more potential against Gram negative bacteria than Gram positive bacteria. *Klebsiella pneumonia* was more susceptible to the hemolymph proteins exhibiting a zone of inhibition measuring 27 mm. The supplement of CUD to the culture media further enriched the antibacterial activity of the hemolymph proteins (29 mm). The SDS-PAGE analysis indicated two different types of clear bands representing proteins of 53 kDa and 19 kDa. Overall, this investigation signified that the microcrustaceans have a defence mechanism hemolymph of *Mesocyclops leuckarti* have a potential agent for novel antibiotics.

1. Introduction

Copepods represent about 20% of the mean annual zooplankton biomass. They are found in abundance in many aquatic ecosystems (Huys and Boxshall, 1991) and conspicuous member of aquatic biota that have adapted to various habitats such as damp moss, interstitial sand, subterranean localities and parasitic living. The cyclopoid copepods are successful inland water group animals and *Mesocyclops leuckarti* is the important planktonic cyclopoid copepods. In evolution, invertebrates have not developed acquired immune systems. However, they have been bestowed with non-specific immune mechanisms. Crustaceans show many antibacterial proteins in their hemolymph. However, the literature does not show any record of anti bacterial proteins in microcrustaceans including zooplankton (Iskratsch et al., 2009; Iwanaga, 1993; Kawabata et al., 1995; Mori and Stewart, 1978; Jayasankar and Subramoniam, 1999) (Table 1).

Hemolymph is the type of blood found in Arthropod's open circulatory system. It contains many bioactive molecules which have functional roles in the defence system. The molecules include lectins, complement, clotting factors, antimicrobial peptides (Vazquez et al., 2009).

Among them the antimicrobial peptides are the prime factors that give immunity to the animal. There are two types of antimicrobial peptides identified in hemolymph. They are high molecular weight large antimicrobial proteins (>100 amino acids) and low molecular weight small antimicrobial proteins. The high molecular weight antimicrobial proteins target the disrupt microbial biomolecules and small antimicrobial proteins disrupt the structure and/or the function of microbial cells (Aspan et al. (1995); Stabili et al. (1999); Fujimoto et al. (1995); Hall et al. (1995)). These antimicrobial peptides are secreted in

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Table 1
Antibacterial activity of high molecular protein in freshwater zooplankton *Mesocyclops leukarti* hemolymph against bacterial pathogens.

Samples	Zone of Inhibition in diameter (mm)			
	Klebsiella pneumonia	*Escherichia coli*	*Shigella flexneri*	*Staphylococcus aureus*
Control	–	–	–	–
S1	27 mm	20 mm	17 mm	19 mm
S2	29 mm	25 mm	20 mm	25 mm
Amikacin (Standard)	27 mm	25 mm	28 mm	30 mm

- = No zone
Control = Distilled water
S1 = Hemolymph of plankton cultured without cow urine distillate
S2 = Hemolymph of plankton cultured with cow urine distillate

hemolymph in response to microbial invasion the structure and or the functions. Similarly, the hemocytes in the hemolymph are called as granulocytes or amoebocytes that contain two types of secretory granules (large and small but dense) Toh et al. (1991). These granules are highly sensitive to bacterial endotoxins. Serine protease zymogens (factor C and factor G) (Muta et al., 1993; Mutaet al., 1993; Mute and Iwanaga,1996) forming the components of granules are auto catalytically activated by LPS and Jayasankar and Subramoniam (1999) β-D glucan present in Gram negative bacteria and fungi and triggers the coagulation cascade. This activation leads to the formation of insoluble coagulin gel from coagulogen and engulf or immobilize the invaded pathogen by forming clot. The entrapped microbe is subsequently killed by lectins and antimicrobial substances released by the granules (Iwanaga, 1993; Iwanaga et al., 2001; Iwanaga et al., 1992).

Recently, exopolysaccharide (Abinaya et al., 2018), selenium nanowires (Abinaya et al., 2019), green symthesized Ag nanoparticles (Jayanthi et al., 2017; Ishwarya et al., 2017; 2019; Al-Ansari., 2019), β-glucan-binding protein (Divya et al., 2020a,2020b), Biopolymer gelatin-coated zinc oxide nanoparticles (Divya et al., 2018), hen's albumen extract (Sherly Carolyn et al., 2019), biopolymer zein-coated gold nanoparticles (Suganya et al., 2017), chitosan-alginate microspheres (Thaya et al., 2018), phytoextracts (Govindarajan et al., 2008; Kolanjinathan et al., 2009) are used for the antibacterial activity.

Though this information is widely available in literature on crustacean immunity, none of the studies has been reported so far on micro crustaceans including Zooplankton. The present investigation was taken up to find proteins from hemolymph of *Mesocyclops leuckarti* and to evaluate their immune potential in terms of antimicrobial activity.

2. Materials and methods

2.1. Collection and maintenance of Mesocyclops leuckarti

Mesocyclops leuckarti were collected from a local freshwater pond in and around Kumbakonam (10.9602° N, 79.3845° E), Thanjavur district, Tamilnadu, India and cultured in our laboratory with 0.025% of cow urine distillate medium.

2.2. Isolation of hemolymph

Using a hand lens 20 mature *Mesocyclops leuckarti* adults were separated and homogenized with sterile physiological saline solution in a tissue homogenizer. The homogenate was centrifuged for 10 min at 600 rpm. The resulting supernatant alone was collected and the cell debris was throughout. The collected supernatant was supposed to be the hemolymph (Praveena and Venkatalakshmi, 2019).

2.3. Estimation of total proteins in hemolymph

Lowry et al. (1951) method with bovine serum albumin as the standard was followed for quantification of proteins in hemolymph samples.

2.4. Molecular weight determination of protein in hemolymph

The purity, homogeneity and molecular weight of proteins obtained from hemolymph of zooplankton, *Mesocyclops leuckarti* was determined by SDS-PAGE consisting of 4% and 12% polyacrylamide in stacking and separating gel respectively. 10 μl of the sample was taken and mixed with 10 μl twofold concentrated buffer. The mixture was heated for 15 min at 70 °C Deraz et al. [2005]. The sample is now loaded in the gel and electrophoresis was carried at a constant voltage of 60 V for two hrs. For determining the molecular weight of the protein bands, molecular mass markers were used. Staining of the gel was done using ethidium bromide.

2.5. In gel digestion

The same process described earlier in followed for digestion process Shevchenko et al. (2007). The gel was placed on a light box and the separated bands were cut separately using a clean sealpel. The excised bands were cut into small cubes measuring 1x1 mm and centrifuged in a micro centrifuge. The gel cubes were washed with destaining buffer (25 mM NH_4HCO_3 dissolved in 50% ethanol) for 20 min at 25° C. The process was repeated until the ethidium bromide stain was taken off. The gel cubes after destaining were dehydrated using 100% acetonitrile for 10 min and rehydrated with 10 mM DTT in 50 mM NH_4HCO_3 (reduction buffer) and incubated for 60 min at 55° C. After incubation the gel cubes were treated with alkylation buffer (50 mM iodoacetamide in 50 mM NH_4HCO_3) and incubated in dark conditions for 45 min at 35° C. Then the gel cubes were washed for 20 min with digestion buffer (50 mM NH_4HCO_3 in H_2O; pH 8.0). Dehydration and washing was repeated continuously for proper drying. The dried gel cubes were coated with trypsin solution and refrigerated at 4° C for 30 min. The gel matrix was further treated with extraction buffer (3% trifluoroacetic acid and 30% acetonitrile) for 15 min to remove the peptides from the gel. After extraction the gels were centrifuged and the supernatant was collected and dried to remove acetonitrile and reacidified with 2% trifluoroacetic acid. The digested peptides were stored at −25° C until further use.

2.6. Antibacterial activity

The antibacterial activity of the hemolymph proteins was determined using the well diffusion method. 25 ml of Muller-Hington agar was poured in petriplates. After optimal temperature is attained, it is inoculated with 0.1 ml of 24 h broth culture of patho-

genic bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Shigella flexneri*) in separate plates. The bacterial inoculums is made to mix uniformly by gentle swirling of the plates. After complete solidification of the agar, three wells of 6 mm diameter were formed using a gel borer. Each well is filled with 50 μl of the protein fraction (low or high molecular weight protein), standard antibiotic amikacin and control was left separately. The inoculated perti plates were then incubated at 30° C for 48hr. The diameter of the inhibition zone was measured using calipers. Each antibacterial assay was performed in triplicate and mean values were reported by McKenna, 2013 and Lila and Tendencia Eleonor, 2004.

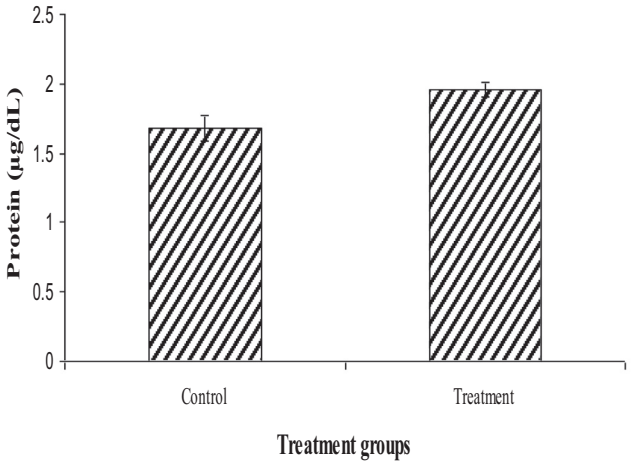
3. Results

The results of fractions by SDS-PAGE indicated the molecular weight of antimicrobial proteins in the hemolymph of *Mesocyclops leuckarti* were approximately in the range between 14 kDa and 100 kDa (Fig. 1). The appearance of single band in each lane in 12% gel indicated their purity.

Fig. 2 depicts the total amount of protein isolated from the hemolymph of the zooplankton *M. leuckarti* that was cultured with the presence and absence of cow urine distillate (CUD) 68 and 96 μg/dl of protein were isolated from the hemolymph of zooplankton cultured with CUD and without CUD respectively (Figs. 3–5).

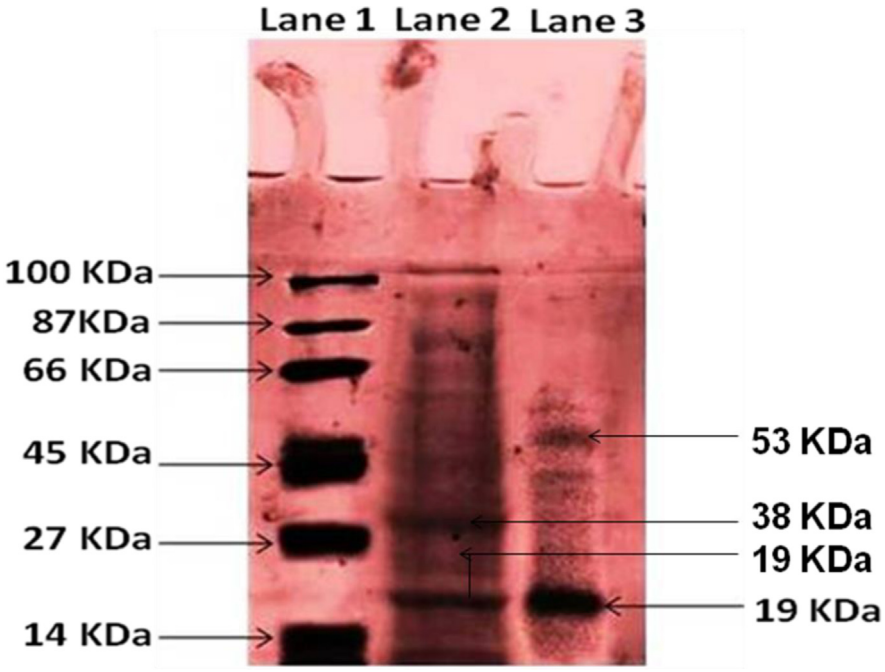
Antibacterial activity of proteins obtained from hemolymph of *M. leuckarti* was assessed by an agar well diffusion method. The results obtained from the experiments conducted are shown in Figures and Tables. From theses results the following facts could be derived. The high molecular weight protein fraction in the hemolymph of *Mesocylops leuckarti* cultured in either in the presence or absence of CUD showed significant antibacterial activity on with the standard antibiotic amikacin against all the human pathogens tested. Among them, the CUD supplementation showed significant impact on the antibacterial activity of the high molecular weight protein fraction of the hemolymph. The maximum activity was shown against *Klebsiella pneumonia* (20 mm) and minimum activity was recorded against *Shigella flexneri* (20 mm) (Figs. 6–8).

The results shown in Table 2, the low molecular weight indicated that the hemolymph samples collected from *Mesocyclops*



Control – Zooplankton culture without Cow urine distillate Treatment – Zooplankton culture with Cow urine distillate

Fig. 2. Protein Estimation of Freshwater Zooplankton Hemolymph and Hemolysate of *Mesocyclops leuckarti*.

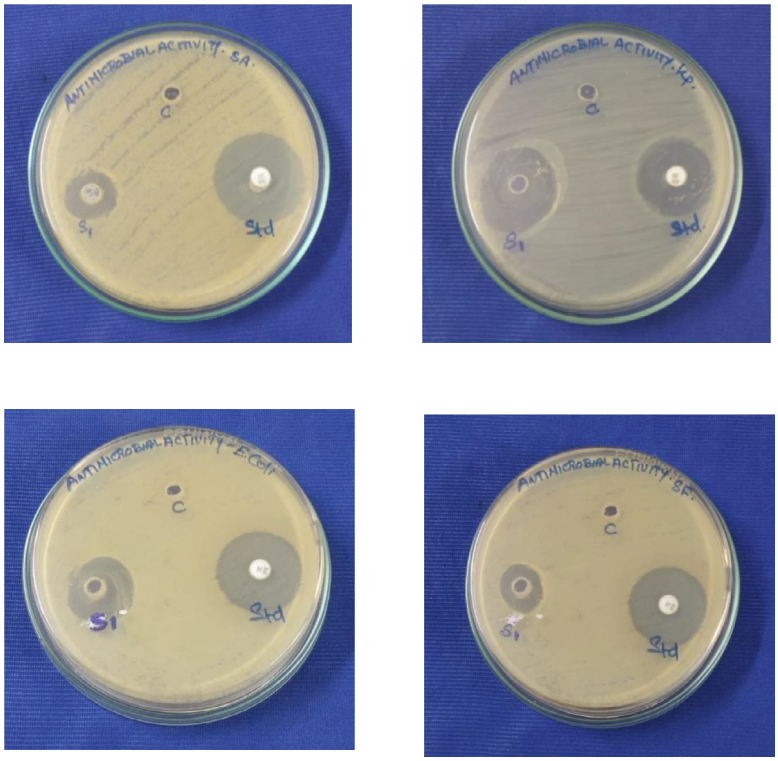


Lane 1 – Marker

Lane 2 – S1 (Hemolymph of plankton cultured without cow urine distillate)

Lane 3 – S2 (Hemolymph of plankton culture with cow urine distillate)

Fig. 1. SDS-PAGE of the identified protein from the hemolymph of *Mesocyclops leuckarti*.



S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shigella flexneri*; S1: Hemolymph of plankton cultured without CUD; CUD: Cow urine distillate

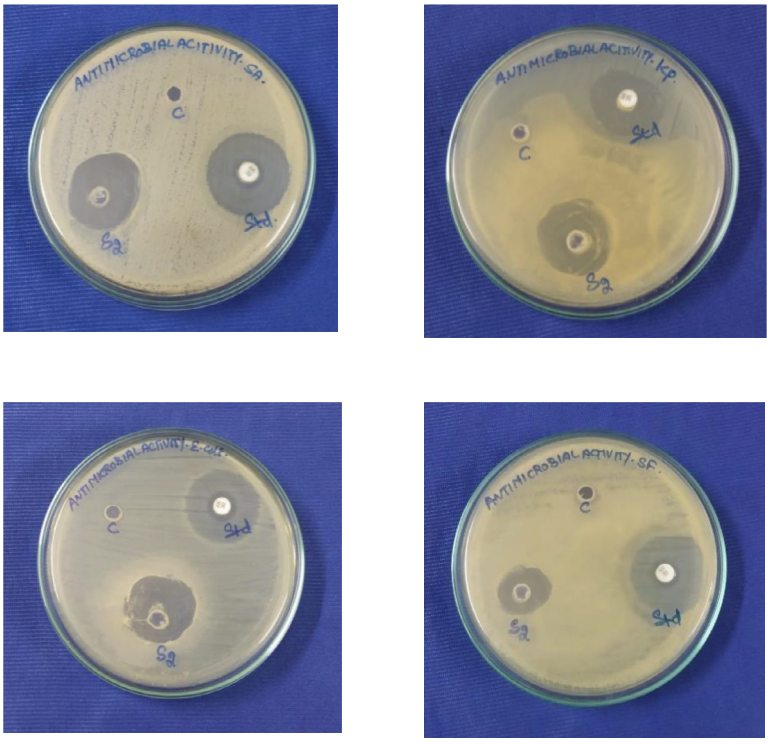
Fig. 3. Antibacterial activity high molecular protein of hemolymph of *Mesocyclops leuckarti* cultured without cow urine distillate.

leuckarti cultured without CUD showed antibacterial activity against *Klebsiella pneumonia* (14 mm) and no activity against other pathogens. However, the *M. leuckarti* organisms cultured in the presence of CUD possessed low molecular weight protein fractions that expressed antibacterial activity against all pathogens with maximum activity against *Klebsiella pneumonia* (18 mm) and minimum activity against *Shigella flexneri* (12 mm) as similar to the activity of high molecular weight protein fraction.

4. Discussion

A number of multidrug resistant bacterial species are listed by WHO that requires the urgent development of novel antimicrobials McKenna (2014). For the first time to the best of our knowledge, an attempt utilizing the antimicrobial proteins derived from the hemolymph of freshwater zooplankton *M. leuckarti* as a substitute for conventional antibiotics for the control of multidrug resistant bacterial isolates. The peptides derived from the hemolymph of invertebrates exhibit a broad spectrum of activities Mona Hajirasouli and Jamileh Pazooki, (2014) and these peptides mainly target the bacterial membrane. With the advent of artificial intelligence and bioinformatics tools unprecedented sequences and sequence combinatorial space of antimicrobial peptides have been generated (Porto, 2018a; Porto et al., 2018b). The present study documented the antimicrobial property of the hemolymph proteins from fresh water zooplankton against selected microbial pathogens. From the results it is evident that the proteins from the hemolymph were effective towards Gram- negative bacterial species compared to Gram positive bacteria. In agreement with our study, the antimicrobial proteins of *Charybdis lucifera* hemolymph exhibited effective antimicrobial activity against the Gram- negative bacteria *E.coli* (11 mm) and *P.aeruginosa* (11 mm) compared to the Gram positive bacteria Rameshkumar et al. (2009).

Several previous literatures reported the positive role of CUD in the treatment of cardiac, respiratory, kidney diseases (Ojewole and Olusi, 1976; Chauhan et al. 2001) and antimicrobial activity Yadav et al. (2008). The present study observed an increase in antibacterial activity of the hemolymph proteins against the selected human pathogenic bacterial species when cultured in the presence of CUD. This enriched antibacterial activity might be due to the fact that CUD itself possesses antibacterial, antifungal and antimicrobial activities (Hu et al. 2007; Shaw et al. 2007) Hence due to these properties, the plankton cultured in CUD media reflected the same properties by imbibing the active ingredients from CUD into hemocoel and hemolymph Badadani et al. (2007). A similar study on the antimicrobial activity of CUD against periodontal pathogens was also reported Gupta et al. (2017) and Bardvalli et al. (2016). Hemolymph of *Maydelliathelphusa masoniana* showed effective antibacterial activity against *Escherichia coli, Streptococcus pyrogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis* and *Salmonella paratyphi* Rakesh et al. (2019). These results indicate that hemolymph have developed a variety of defense molecules against pathogenic microbes, but the degree of antimicrobial activity may vary depending upon the species Ojewole and Olusi (1976). It was reported in anearlier study that the concentration of hemolymph proteins exhibits wide inter specific variations and hemocytes might be the site of production and storage of these antimicrobial peptides. In the present study high molecular weight proteins of hemolymph exhibited effective antimicrobial property against the selective microbial pathogens



S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shigella flexneri*; S2: Hemolymph of plankton cultured with CUD; CUD: Cow urine distillate

Fig. 4. Antibacterial activity high molecular protein of hemolymph of *Mesocyclops leuckarti* cultured with cow urine distillate.

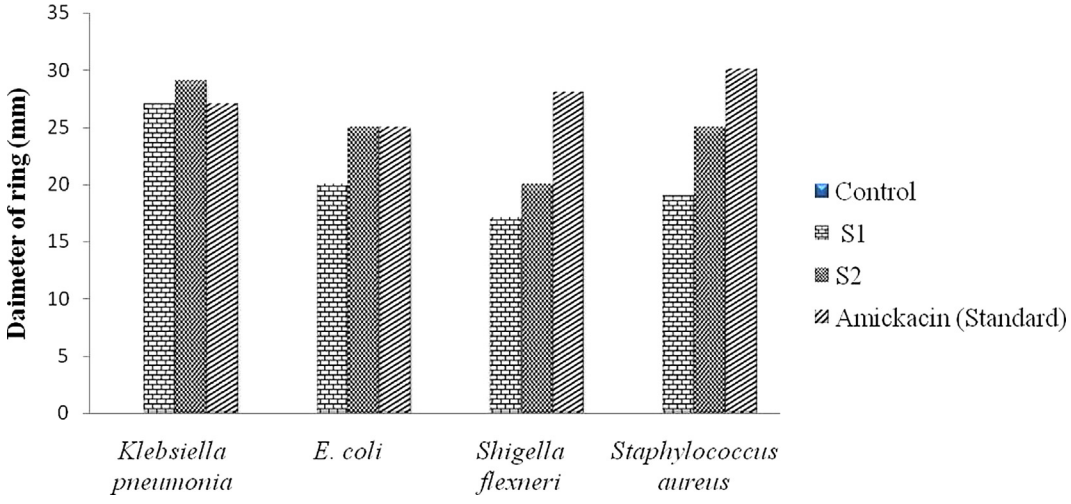
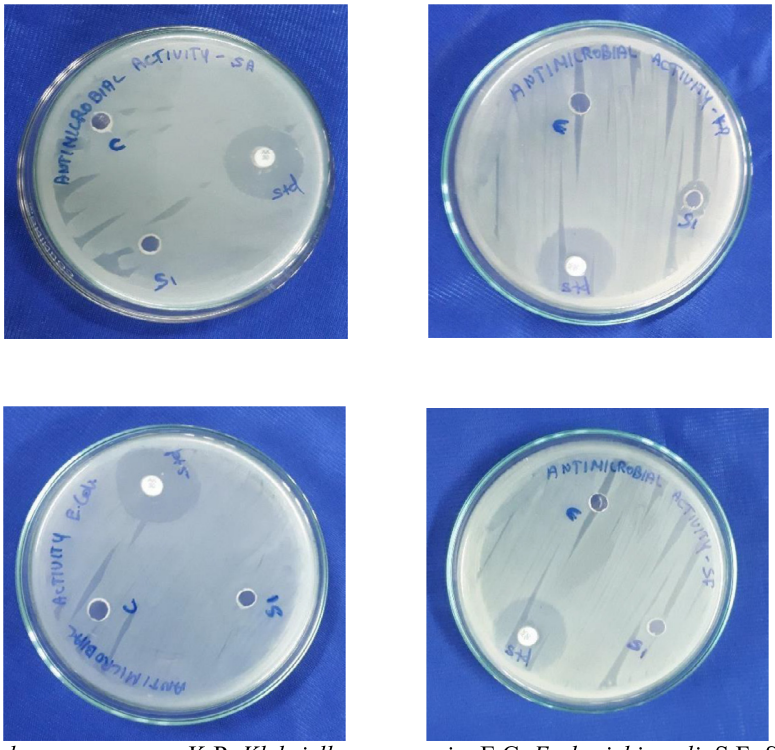


Fig. 5. Antibacterial activity of high molecular protein in hemolymph of *Mesocyclops leuckarti.*

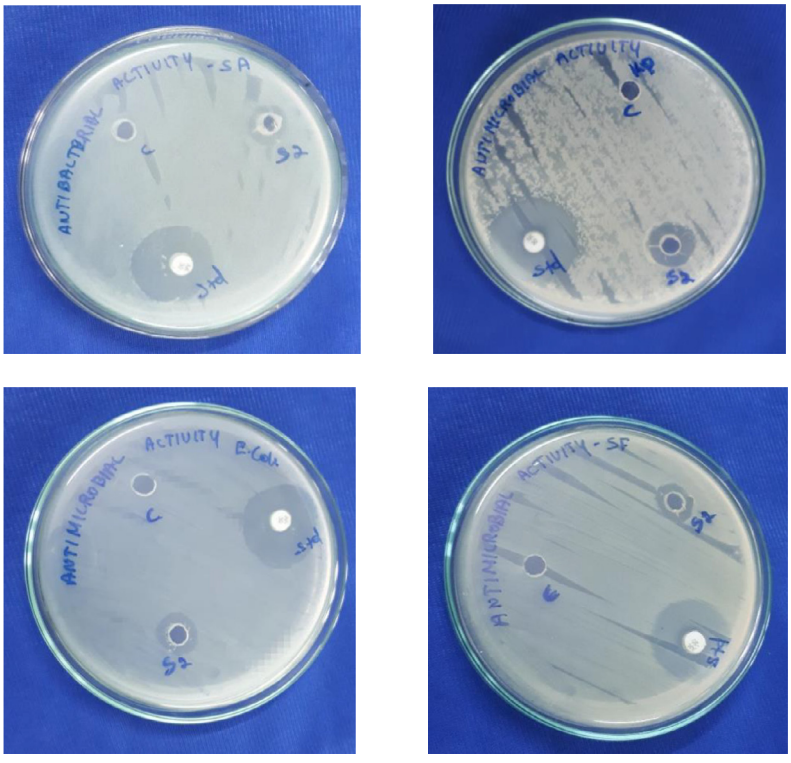
compared to low molecular weight proteins of hemolymph. The supplement of CUD in the growth media further enhanced the antagonistic activity of the hemolymph proteins against microbial pathogens. Thus the present investigation indicates that the hemolymph proteins of *M. leuckarti* may contain potential antibiotics. The antimicrobial assay performed in this study may form a baseline information for further studies revealing the fact that *M. leuckarti* will provide an opportunity for the production of new natural alternatives for antibiotics

In conclusion, the present study reported the isolation of hemolymph proteins from the fresh water zooplankton *M. leuckarti* and evaluated its antagonistic activity against human pathogenic bacteria. Hemolymph proteins can be used as an alternative to conventional antibiotics since there is a global concern on the antibiotics overuse or misuse. Several issues have to be dwelt before the application of hemolymph proteins as antimicrobials such as production cost, in vivo efficacy, frequency of application and dosage etc. Overall, this study might be a promising solution



S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shigella flexneri*; S1: Hemolymph of plankton cultured without CUD; CUD: Cow urine distillate

Fig. 6. Antibacterial activity low molecular protein of hemolymph of *Mesocyclops leuckarti* cultured without cow urine distillate.



S.A: *Staphylococcus aureus;* K.P: *Klebsiella pneumonia;* E.C: *Escherichia coli;* S.F: *Shigella flexneri*; S2: Hemolymph of plankton cultured with CUD; CUD: Cow urine distillate

Fig. 7. Antibacterial activity low molecular protein of hemolymph of *Mesocyclops leuckarti* cultured with cow urine distillate.

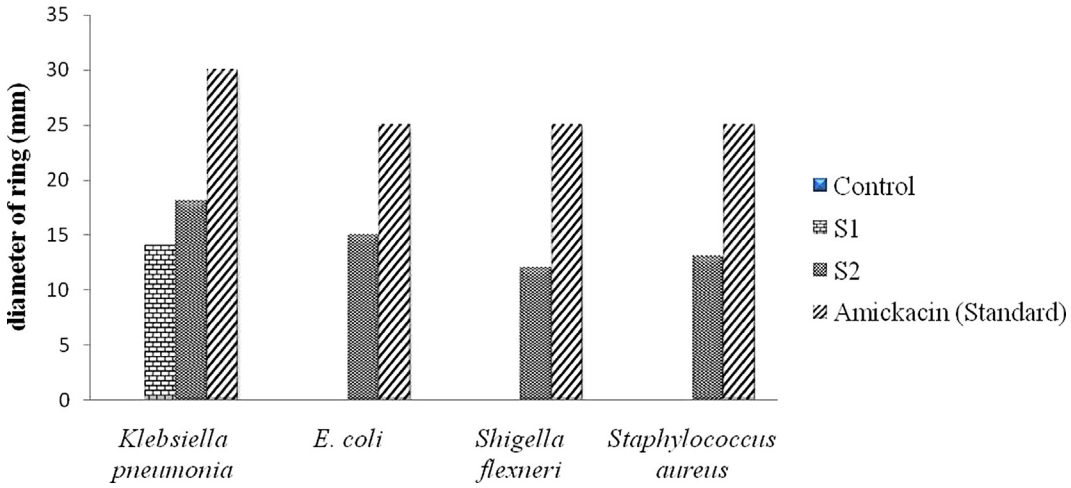


Fig. 8. Antibacterial activity of low molecular protein in hemolymph.

Table 2
Antibacterial activity of low molecular protein in freshwater zooplankton *Mesocyclops leuckarti* hemolymph against bacterial pathogens.

Samples	Zone of Inhibition in diameter (mm)			
	Klebsiella pneumonia	*Escherichia coli*	*Shigella flexneri*	*Staphylococcus aureus*
Control	–	–	–	–
S1	14mm	–	–	–
S2	18mm	15mm	12mm	13mm
Amikacin (Standard)	30mm	25mm	25mm	25mm

- = No zone
Control = Distilled water
S1 = Hemolymph of plankton cultured without cow urine distillate
S2 = Hemolymph of plankton cultured with cow urine distillate

for the production of alternative antimicrobial agents against drug resistant bacterial strains.

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