

# A Study On The Antibacterial Activity In The Haemolymph Of Larvae Of The Mulberry Silkworm, *Bombyx Mori L.* In Response To Injection Of The Bacteria, *Bacillus Cereus*

Ponmurugan, M. Karthikeyan, A

**Abstract:** The antibacterial activity of haemolymph of the induced fifth instar larvae of the mulberry silkworm, *Bombyx mori* in response to *Bacillus cereus* was investigated by well diffusion method. The control larvae C1 were fed with normal leaves till cocoon spinning. The second set of control larvae C2 were injected with bacteria broth solution. The experimental larvae E1 were injected with live bacteria *B. cereus*, E2 were injected with attenuated bacteria, E3 were injected with a mixture of haemolymph derived from E1 larvae and live bacteria and E4 were injected with a mixture of haemolymph derived from E2 larvae and live bacteria. Zone of inhibition for the haemolymph of E1 and E2 against *E. coli* was 6mm and 5mm respectively. Likewise zone of inhibition of E1 and E2 was 7mm and 5mm respectively against *B. cereus*. Only minimum zone of inhibition of 2mm and 1mm was observed against both *Staphylococcus aureus* and *Klebsiella pneumonia* respectively. It was inferred that *Escherichia coli* and *B. cereus* were sensitive to haemolymph of E1 and E2. Purified haemolymph proteins exhibited a moderate zone of inhibition of 3mm against all the four bacteria tested. Compared to crude haemolymph purified sample of E2 showed greater antibacterial effect against *Staphylococcus aureus* and *Klebsiella pneumonia*.

**Key words:** Zone of inhibition, Haemolymph, Antibacterial activity, Attenuated bacteria

## 1. INTRODUCTION

One of the successful species in evolution are insects. Insects are continuously exposed to pathogenic microorganisms and are able to defend against infection [1]. Generally, an effective immune response involves three steps, recognizing the pathogen, signaling to effectors, and a defensive response [2]. In insects which lack an adaptive immune system, antimicrobial peptides play a crucial role in fighting the invading pathogens. Silkworm *B. mori* is being used as an animal model as it has both cellular and humoral immune system which together form a potent defense mechanism against invading bacteria [3]. Insect defensins are active mainly against Gram-positive bacteria, including *Micrococcus luteus*, *Aerococcus viridians*, *B. megaterium*, *B. subtilis*, *B. thuringiensis* and *S. aureus*. Some insect defensins are also active against Gram-negative *E. coli* and some fungi [4]. The aim of this work was to study antibacterial activity of antibacterial proteins in the haemolymph of the larvae of silkworm after injection of bacteria *B. cereus* and to analyse the antibacterial activity of crude haemolymph against the bacteria *E. coli*, *Klebsiella pneumonia*, *S. aureus* and *B. cereus*.

## 2. MATERIALS AND METHODS

### Rearing of *Bombyx mori L.* larvae

The commercial breed of the mulberry silkworm i.e., the double hybrid CSR6 × CSR 26 × CSR2 × CSR27 was used for the study. Newly hatched first instar larvae were reared upto the end of fourth instar under the standard temperature of 25 to 28°C and humidity of 75-80% by shelf rearing method suggested by Krishnaswamy et al (1978) [5].

### Infection of silkworm larvae with bacterial strain

Three hundred freshly moulted fifth instar silkworm larvae were divided in to six groups (2- Control and 4- Experimental sets), each group consisting of 50 larvae. The control larvae (C1) were fed with normal leaves till cocoon spinning. The second set of control larvae (C2) were injected with bacterial broth solution. The experimental larvae E1 were injected with broth containing live bacteria *B. cereus* ( $1.1 \times 10^6$  cells/ml); E2 were injected with broth containing attenuated bacteria ( $1.1 \times 10^6$  cells/ml); E3 were injected with broth containing a mixture of haemolymph derived from E1 larvae and live bacteria ( $1.1 \times 10^6$  cells/μl) and E4 were injected with broth containing a mixture of haemolymph derived from E2 larvae and live bacteria ( $1.1 \times 10^6$  cells/μl).

### Haemolymph sample collection

After 24 hours post infection, mortality of healthy and infected larvae were recorded. Live silkworm larvae in all control and experimental sets were used for collection of haemolymph samples. Disinfection was done with 70% ethanol before haemolymph collection. First abdominal leg of larva was cut by using a forceps and free flowing haemolymph was collected into pre-chilled eppendorf tube containing a few crystals phenylthiourea @1mg/sample [6]. Phenylthiourea was used to avoid the activity of prophenoloxidase followed by melanisation of the

- *Research Scholar, PG & Research Department of Zoology, Government Arts College (Autonomous), Karur, Tamil Nadu, India, PH- 9788839200, E-mail: pon.murugan04@gmail.com.*
- *Associate Professor, PG & Research Department of Zoology, Government Arts College (Autonomous), Karur, Tamil Nadu, India*
- *PH- 9443593724, E-mail: prikaravi@gmail.com*

haemolymph samples. The samples were stored at  $-20^{\circ}\text{C}$  for further use.

### Antibacterial Assay

Antimicrobial activity was done by using well diffusion method using Mueller Hinton Agar (MHA) medium and it is prepared as per the standard and sterilized in an autoclave at  $121^{\circ}\text{C}$  for 15 minutes. After sterilization media was poured to sterilized petriplate and allowed for solidification in aseptic condition.  $200\mu\text{l}$  of the seed culture of *E. coli*, *B. cereus*, *S. aureus* and *K. pneumonia* were poured and spreaded on the solidified agar plate with sterile cotton swab and allowed to dry for 2-3 minutes. Wells (10mm) were made with sterile cork borer and  $50\mu\text{l}$  of the control and immunized haemolymph samples were added in each well, erythromycin ( $5\mu\text{g}$ ) was used as a standard positive control and the plates were incubated at  $37^{\circ}\text{C}$  for 24hrs. After incubation period the zone of inhibition was measured in mm and compared with that of the control [7].

### Purification of protein

The obtained protein was purified using Sephadex G75 column (20X4cm). The prewashed column was packed. Sample 4ml was added to the column and equilibrated in 0.3M ammonium acetate at a flow rate of 2ml/10min. The column was washed with 0.3M ammonium acetate, 7.0 pH for 5 times and eluted stepwise. The absorbance of the fractions was measured at 280nm (ELICO SL 159 Microprocessor Spectrophotometer). The fractions showing maximum absorbance was further purified. The above protein was further purified using Sephadex G 100 column (20X4cm) equilibrated with 0.1M phosphate buffer with flow rate of 2ml/15min. The absorbance of the eluted fractions were measured at 280nm and the best were selected for the antibacterial activity as mentioned.

## RESULTS AND DISCUSSION

### Mortality

No mortality was observed in untreated control (C1), very less (4%) mortality was observed in broth injected larvae (C2). The mortality percentage was found to be higher (80%) in larvae injected with E1 and lower (62%) in E2 larvae. 72% and 68% of mortality were observed in E3 and E4 respectively. Many bacteria such as *Bacillus cereus*, *B. cubonius*, *B. bombysepticus*, *B. mycoides*, *B. leterosporus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* have been reported to cause high percentage of mortality due to septicemia and flacherie [8].

### Antibacterial effect of crude haemolymph

Discs impregnated with erythromycin solution was used as positive control to conform the clear zone near by the wells as zone of inhibition of bacterial growth. These discs exhibited a moderate zone of inhibition i.e., 3mm against the two gram positive bacteria, *E. coli* and *K. pneumonia* and two gram negative bacteria *S. aureus* and *B. cereus* (Table 2). The results revealed that crude haemolymph of all experimental samples exhibited greater antibacterial effect than the uninduced control and broth injected control larva against the *B. cereus* bacterial culture. This could be evidenced from the greater zone of inhibition (Table 2 and 3). The zone of inhibition against *B. cereus* was highest in

E1 followed by E3, E2 and E4. The zone of inhibition against *E. coli* was maximum in E1 and E2 than E3, E4 and control. The zone inhibition for the experimental sample against *S. aureus* and *K. pneumoniae* was very low. This shows that the bactericidal effect against *S. aureus* and *K. pneumoniae* was not as good as against *E. coli* and *B. cereus*. The haemolymph of uninoculated larvae (C1) did not exhibit any antibacterial effect against *S. aureus* and *K. pneumoniae*. Likewise, its effect against *E. coli* and *B. cereus* was also very less. The antibacterial effect of the haemolymph of broth injected larvae (C2) was also almost the same as that of C1. The non-induced haemolymph did not show considerable inhibitory activity against any of the tested bacterial strains. It does not indicate that defensive peptides are absent but they may be present in lesser quantity so that no detectable action is seen in vitro studies as reported by Faulhaber and Karp (1992) [9]. It indicates that pathogenic induction or entry may be necessary for the production of additional factors required for the activation of antimicrobial proteins to show their antimicrobial activity. Normal haemolymph of various insects has been reported to kill some kinds of bacteria [10]. It is also well known that a transient bactericidal activity can be readily induced by injecting bacterial vaccine, either specific or unrelated, endotoxin, or sometimes even saline into the larvae or pupae when little or no bactericidal activity is observed in the normal, non vaccinated haemolymph [11]. The two factors are required for killing *E. coli*. One is the lysozyme-like enzyme, other factors such as lysozyme and cofactor might be essential for antibacterial activity. Faye et al (1975) [12] showed that three factors synthesized de novo after the inducing injection of vaccine were necessary for humoral antibacterial activity of the silk moth *Samia cynthia*. Chadwick (1970) [13] showed that the factor in wax moth *Galleria mellonella*, larval haemolymph that kills *Pseudomonas aeruginosa* can be induced rapidly by injection of homologous vaccine. The lysozyme concentration increases parallel to the rise in bactericidal activity, but remains at a high level even after the bactericidal activity has disappeared [13]. Kawarabata (1970) [10] found that bactericidal activity was enhanced significantly by injection of bacterial vaccines, whereas the lysozyme level in the haemolymph was not affected by the vaccination. Powning and Davidson (1973) [14] also reported that bacteriolytic activity was induced in the haemolymph of wax moth and silkworm pre pupae after injection of bacterial vaccine. They showed that lysozyme and cofactor are also responsible for bactericidal activity of haemolymph. Haemolymph provides various kinds of protection and defense from (i) physical injury; (ii) the entry of disease causing organisms, parasites, or other foreign substances; and sometimes (iii) the actions of predators. Injury to the integument elicits a wound-healing process that involves haemocytes and plasma coagulation. A haemolymph clot is formed to seal the wound and reduce further haemolymph loss and bacterial entry. If disease organisms or particles enter an insect's body, then immune responses are invoked. This was evidenced from the greater zone of inhibition in the experimental samples of the present work. Crude haemolymph of larvae inoculated with the live bacteria (E1) exhibited greater antibacterial effect against *B. cereus*, the bacteria that was inoculated and against *E. coli*. The antibacterial effect of crude

haemolymph of the larvae injected with attenuated bacteria (E2) was also almost similar to that of live bacteria injected larvae (E1). However, the zone of inhibition of E2 against the four bacteria tested was little less than that of E1. Similarly *S. aureus* and *K. pneumoniae* were less sensitive to the crude haemolymph of both E1 and E2 larvae. The noticeable feature here is even the attenuated bacteria could induce the production of antibacterial factors. Hence such factors can be isolated and effectively used to kill the pathogens that cause infectious diseases in human beings after an in-depth study. Studies on immune response of the silkworm can be carried out with the attenuated bacteria which are safer than the live bacteria. Another most interesting feature is haemolymph of both E1 and E2 exhibited antibacterial effect against gram negative bacteria *E. coli* though the larvae were inoculated with gram positive bacteria *B. cereus*. E3 and E4 larvae were injected with live bacteria mixed with the haemolymph derived from the live bacteria injected larvae (E1) and attenuated bacteria injected larvae (E2) respectively. The crude haemolymph collected from E3 larvae showed greater antibacterial effect against *B. cereus* and moderate antibacterial effect against *E. coli*. Similarly the crude haemolymph collected from E4 also showed moderate antibacterial effect against *E. coli* and *B. cereus*. However *S. aureus* and *K. pneumoniae* were not at all sensitive to the crude haemolymph of the larvae of E3 and E4. Since the antibacterial effect of crude haemolymph of E3 and E4 was not as good as that of E1 and E2, the haemolymph of E1 and E2 only were purified, proteins were isolated and such protein samples were screened for antibacterial effect.

#### **Antibacterial effect of purified haemolymph proteins**

The antibacterial effect of purified protein samples of haemolymph of control and experimental larva revealed that both E1 and E2 exhibited maximum zone of inhibition against *K. pneumoniae* and moderate against *E. coli*, *S. aureus* and *B. cereus*. Seufi et al (2009) [15] reported that fractionated immunized haemolymph exhibited higher antimicrobial activity whereas in this study purified haemolymph protein samples of E1 and E2 were found to have greater antibacterial effect against *K. pneumoniae* and *S. aureus* and lower antibacterial effect against *B. cereus* and *E. coli* than crude haemolymph. Purified haemolymph protein samples of uninoculated larvae (C1) also showed little or moderate bactericidal effect against all the four tested bacteria. This revealed that some bactericidal proteins were produced naturally in *B. mori*. Surprisingly it was observed that all the four bacteria tested were sensitive to purified haemolymph protein samples of live bacteria injected larvae (E1) and attenuated bacteria injected larvae (E2). *S. aureus* and *K. pneumoniae* which were less sensitive to crude haemolymph of E1 and E2, showed little higher sensitivity to their purified haemolymph protein samples. This revealed that some molecules or elements or factors in the crude haemolymph would have inhibited the binding of antibacterial proteins to the surface of *S. aureus* and *K. pneumoniae* to some extent. This might be due to the presence of some ionic salts such as NaCl, KCl, MgCl<sub>2</sub>, NaNO<sub>3</sub> and Na<sub>2</sub>SO<sub>4</sub> in the crude haemolymph as reported by Christensen et al (1988) [16]. The decrease in activity caused by salt was not due to osmotic stabilization of the bacteria but was due to

inhibition of the adsorption of the peptides to the bacterial surface, which is mediated by ionic interactions [16].

#### **Comparison of crude haemolymph and purified haemolymph**

The purified haemolymph proteins were active against the gram negative *S. aureus* and gram positive *K. pneumoniae* bacteria which were not more susceptible to crude haemolymph. So antibacterial activity is not related to cell wall of bacteria. This contradicts the report of Basseri et al (2016) [17] that the antibacterial activity of the peptides is related to the cell wall of the bacteria. It may be assumed that the proteins identified in this study might play an important role in their self-defense against bacterial infection in American cockroaches individually or cooperatively. The crude haemolymph of immunized silkworm showed better antibacterial activity against *E. coli* and *B. cereus* whereas purified protein samples of haemolymph showed lesser antibacterial activity. This revealed that the antibacterial proteins are not the only factors that are directly involved in killing the bacteria and some other molecules might also be involved in neutralizing the bacteria as suggested by Basseri et al (2016) [17]. Injection of the bacteria elicited the synthesis of a number of peptides and proteins, which are individually or cooperatively active against the foreign microorganisms [18]. The differences in the zone of inhibition of crude haemolymph and purified haemolymph protein samples indicated that some constitutive proteins might also be involved in killing the bacteria. Quantitative increase in protein level of the haemolymph after injection of bacteria suggested the induction mechanism in synthesis of inducible proteins involved in killing the bacteria constitutive inducible proteins act as signaling molecules such as lysosome [19]. Comparatively, *E. coli* and *B. cereus* were more sensitive to crude haemolymph of E1 and E2 than to their purified haemolymph protein samples. This shows that some factors in the crude haemolymph might have augmented the effect of antibacterial proteins to combat bacteria. Otherwise some factors in the crude haemolymph might have opsonised the bacteria so that the antibacterial proteins can act upon the bacteria easily. This can be related with report of Basseri et al (2016) [17] that the antibacterial proteins are not directly involved in killing the bacteria and some other molecules like lysozyme may be involved in signaling mechanism to remove the bacteria. Such proteins already produced naturally in the haemolymph might be of constitutive proteins as interpreted by Bosco-Drayon et al (2012) [19]. Although, whole haemolymph of immune larvae showed antibacterial activity against *E. coli* and *B. cereus* but we could find some what lesser activity in purified protein samples. So this suggests that antibacterial proteins are not directly involved in killing *E. coli*, and some molecules may be involved in signaling mechanism to remove the bacteria. Adamo (2004) [20] stated that bacteria injected haemolymph have high protein concentration because of induced proteins formed against bacteria for self defense and survivability. Seufi (2011) [15] showed that the various bacteria such as *S. aureus*, *Streptococcus* and *E. coli* bacteria challenged *Spodoptera litura* larvae had variations in the protein profile of its haemolymph. Studies with *B. mori* and *Maduca sexta* haemolymph had revealed that the presence of a variety of

proteins formed in response to injury or bacterial challenge [3]. Gajandra et al (2011) [21] reported that the greater synthesis of protein profile from fat body and release into the haemolymph of silkworm, *Antheraea mylitta* larvae during bacterial infection. The bacterial inoculated haemolymph of Eri silkworm, antibacterial activity was observed higher after post inoculated larval haemolymph obtained from *E. coli* and *M. luteus* bacteria challenged insects. Sharma et al (2005) [22] also noticed that the increased level of antibacterial activity in muga silkworm haemolymph after bacterial challenge. However, Chapelle et al (2009) [23] suggested that the increased level of antibacterial activity was time dependant in *E. coli* and *M. luteus* challenged haemolymph of *S. litura* and *S. frugiperda*. Similarly, Wojda et al (2009) [24] also recorded an increase of antibacterial activity level in cell free haemolymph obtained from *G. mellonella* larvae [25]. In the *S. frugiperda* unchallenged larvae and sterile injured larvae haemolymph had antibacterial activity against gram positive *M. luteus* bacteria and it was reported by Chapelle et al (2009) [23]. The proteins and peptides pattern by gel electrophoresis of haemolymph samples collected 24 hpi revealed the induction of six immune proteins and peptides i.e. ApoL P-I & II, transferrin, defensin, leucocine and major royal jelly protein (MRJP). None of these Antimicrobial peptides (AMP) were detected in haemolymph samples collected from non infected larvae Basseri et al [17].

## CONCLUSION

The haemolymph of live bacteria injected bacteria and attenuated bacteria injected bacteria exhibited greater antibacterial effect against the gram negative *E. coli* and gram positive *B. cereus*. The haemolymph of larvae immunized with live bacteria (E3) exhibited greater antibacterial effect against *B. cereus* whereas haemolymph of larvae immunized with attenuated bacteria (E4) exhibited only moderate antibacterial effect against *B. cereus*. The purified haemolymph proteins samples of larvae immunized with attenuated bacteria exhibited greater potential antibacterial effect than that of the haemolymph of larvae immunized with live bacteria. Comparatively crude haemolymph was having better antibacterial effect than the purified proteins against some bacteria and vice versa.

## ACKNOWLEDGEMENTS

We wish to express sincere thanks to the Principal and Head and other Professors, Lab assistant and Research scholars of the PG and Research Department of Zoology of Government Arts College (Autonomous), Karur, Tamilnadu, for providing the laboratory facilities to carry out this research work.

## REFERENCES

- [1] J.A. Hoffman, "Innate immunity of insects", *Curr Opin Immunol*, 7: 4-10, 1995.
- [2] J.A. Hoffmann, F.C. Kafatos, C.A. Janeway and R.A. Ezekowitz, "Phylogenetic perspectives in innate immunity", *Science*, 284: 1313-1318, 1999.
- [3] E.G. Abraham, J. Nagaraju, D. Salunke, H.M. Gupta and R.K. Datta, "Purification and partial characterization of an induced antibacterial protein in the silkworm", *Bombyx mori*, *J Invert Pathol*, 65: 17-24, 1995.
- [4] H.Y. Yi, M. Chowdhury, Y.D. Huang and X.Q. Yu, "Insect antimicrobial peptides and their applications", *Appl Microbiol Biotechnol*, 98(13): 5807-5822, 2014.
- [5] S. Krishnaswamy, "New technology of silkworm rearing", Central Sericultural Research and Training Institute, Central Silk Board, India, *Bulletin*, (2): 1-23, 1978.
- [6] J.E. Silva, B. Boleli and I.C. Simoes, "Haemocyte types and total and differential counts in unparasitized and parasitized *Anastrepha obliqua* (Diptera, Tephritidae) larvae", *Braz J Biol*, 62: 4, 2001.
- [7] D. Hoffmann, D. Hultmark and H. Bomann, "Insect immunity *Galleria mellonella* and other Lepidoptera have *cecropia* P9, Like factors active against gram negative bacteria", *Insect Biochemistry*, 11: 537-548, 1981.
- [8] M.M. Rai, D.G. Gore, M.K. Rathod, A.M. Khurad, "Evidence of transovarial transmission of *Bacillus subtilis* in the silkworm, *Bombyx mori* L", *J Pharm res*, 7: 318-323, 2013.
- [9] L.M. Faulhaber, R.D. Karp, "A diphasic immune response against bacteria in the American cockroach", *Immunology*, 75: 378-381, 1992.
- [10] T. Kawarabata, "Studies of an acquired resistance on microbial infections in the silkworm (*Bombyx mori* L)", *Sci Bull Fac, Agric Kyushu Univ*, 24: 231-254, 1970.
- [11] H.G. Boman, I. Nilsson-Faye, K. Paul and T. Rasmuson, "Insect immunity Characteristics of an inducible cell-free antibacterial reaction in haemolymph of *Somiacynthia pupae*", *Infect Immun*, 10: 136-145, 1974.
  - I. Faye, A. Pye, T. Rasmuson, H.G. Boman, I.A. Boman, "Insect immunity, Simultaneous induction of antibacterial activity and selection synthesis of some hemolymph proteins in diapausing pupae of *Hyalophora cecropia* and *Samia Cynthia*", *Infect Immun*, 12: 1426-1438, 1975.
- [12] J.S. Chadwick, "Relation of lysozyme concentration to acquired immunity against *Pseudomonas aeruginosa* in *Galleria mellonella*", *J Invert Pathol*, 15: 455-456, 1970.
- [13] R.F. Powing, W.J. Davidn, "Studies on insect Bacteriolytic enzymes, Lysozymes in haemolymph of *Galleria mellonella* and *B. mori*", *Comp Biochem Physiol*, 45: 669-686, 1973.
- [14] A.M. Seufi, E.E. Hafez and F.H. Galal, "Identification, phylogenetic analysis and expression profile of an anionic insect defensin gene, with antibacterial activity, from bacterial challenged cotton leafworm, *Spodoptera littoralis*", *BMC Mol Biol*, 12: 47-51, 2011.

[15] B. Christensen, J. Fink, R.B. Merrifield and D. Mauzerall, "Proc Natl Acad Sci", USA, 85: 5072-5077, 1988.

[16] H.R. Basseri, A.D. Khoeni, R. B. Mandan, A.R Hajhosseini-Baghdadabadi, "Isolation and purification of an antibacterial protein from immune induced haemolymph of American cockroach, *Periplaneta Americana*", J Arthropod-Borne Dis, 10(4): 519-527, 2016.

[17] Vilcinskas, "Evolutionary plasticity of insect immunity", J Insect Physiol, 59: 123-129, 2013.

[18] V. Bosco-Drayon M. Poidevin, I.G. Boneca, K. Narbonne-Reveau, J. Royet and B. Charroux, "Peptidoglycan sensing by the receptor PGRP-LE in the *Drosophila* gut induces immune responses to infectious bacteria and tolerance to microbiology", Cell Host Micro, 12: 153-165, 2012.

[19] S.A. Adamo, "Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*", J Insect Phy, 50: 209-216, 2004.

[20] P.S. Ganjendra, S. Ajit Kumar, D.K. Roy, S. Alok, N. Kallahally, P. Madhusudhan and K. Kiran, "Cellular and Biochemical changes of *Antheraea mylitta* D, on immunization with Attenuated *Antheraea mylitta* Cytoplasmic Polyhedrosis Virus", Intr J Zool Res, 7 (3): 263-271, 2011.

[21] J.A. Sharma, B. Yadav, G. Unni and M.C. Kalita, "Antibacterial proteins from non mulberry silkworms against flacherie causing *Pseudomonas aeruginosa* AC3", Current Science, 89: 9-10, 2005.

[22] M. Chapelle, P.A. Girard, F. Cousserans, N.A. Volkoff and B. Duvic, "Lysozymes and lysozyme-like proteins from the fall armyworm, *Spodoptera frugiperda*", Mol Immunol, 47: 261-9, 2009.

[23] Wojda, P. Kowalski, and T. Jakubowicz, "Humoral immune response of *Galleria mellonella* larvae after infection by *Beauveria bassiana* under optimal and heat shock conditions", J Insect Physiol, 55: 525-531, 2009.

[24] J. Jarosz, "Haemolymph immune proteins protect the insect body cavity from invading bacteria", Comp Biochem Physiol, 111: 213-220, 1995.

**Table 2** - Antibacterial effect of crude haemolymph of control and experimental larvae of the mulberry silkworm, *B. mori*

Organism	Sample (zone of inhibition mm)						
	C1	C2	E1	E2	E3	E4	Disc
<i>E. coli</i> (G)	1	1	6	5	3	3	3
<i>K. pneumoniae</i> (G-)	0	0	2	1	0	0	3
<i>S. aureus</i> (+)	0	0	2	1	0	0	3
<i>B. cereus</i> (G+)	1	0	7	5	6	4	2

**Table 3** - Antibacterial effect of purified haemolymph protein samples of control and experimental larvae of the mulberry silkworm, *B. mori*

Organism	Sample (zone of inhibition mm)			
	C1	E1	E2	Disc
<i>E coli</i> (G-)	2	4	5	3
<i>K. pneumoniae</i> (G-)	3	5	6	3
<i>S. aureus</i> (G+)	2	4	5	3
<i>B. cereus</i> (G+)	3	4	3	4

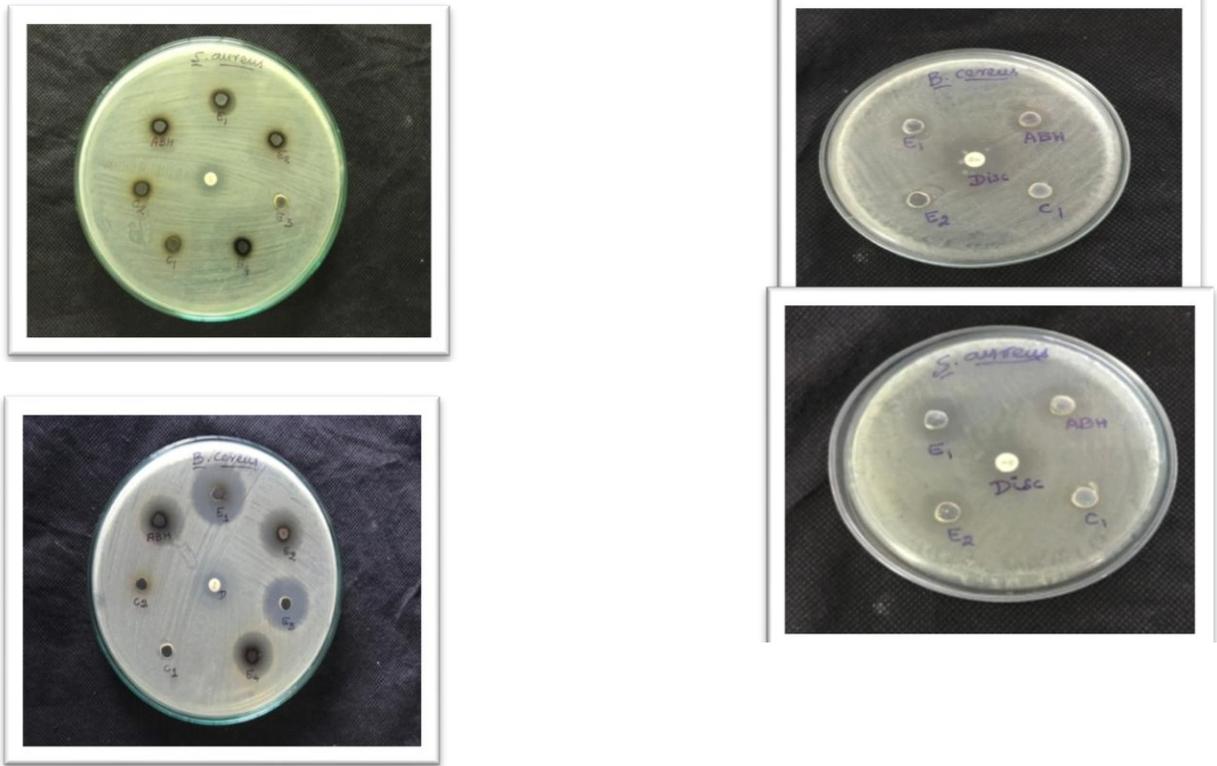
Plate 1 - Antibacterial activity of crude haemolymph of control and experimental larvae against *E. coli* and *K. pneumoniae*



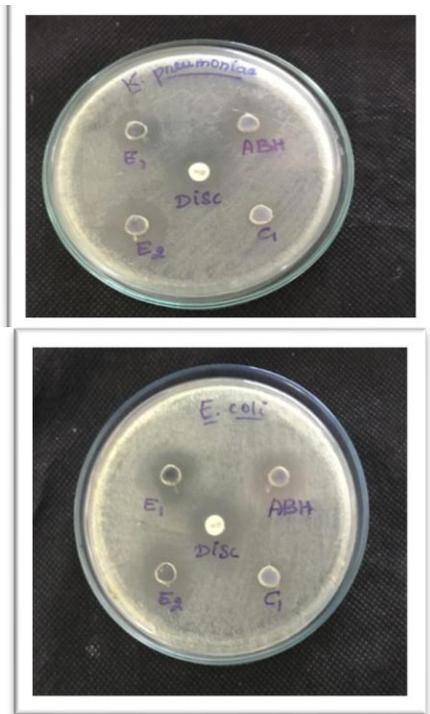
**Plate 2** - Antibacterial activity of crude haemolymph of control and experimental larvae against *S. aureus* and *B. cereus*

**Table: 1** Percentage of mortality of fifth instar larvae of the mulberry silkworm *Bombyx mori* L. in response to the injection of *Bacillus cereus*

Experimental Group	Cumulative % of mortality
C1	0
C2	04%
E1	80%
E2	62%
E3	72%
E4	68%



**Plate 3** - Antibacterial activity of purified haemolymph of control and experimental larvae against *K. pneumonia* and *E. coli*



**Plate 4**- Antibacterial activity of purified haemolymph of control and experimental larvae against *B. cereus* and *S. aureus*