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Investigation into the antimicrobial property of methanol-extracted hemolymph proteins of immunized fifth instar larvae of eri silkworm *Philosamia ricini* (Lepidoptera: Saturniidae)

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Abstract

Fifth instar larvae of Eri silkworm, *Philosamia ricini* were grouped as control (C) - injected with Phosphate Buffer Saline (PBS) and Infected (I)- injected with a bacterial suspension of *Escherichia coli* to induce innate immune responses. After stipulated time-intervals of 24 (I-24) hours and 48 hours of injection (I-48), hemolymph samples were collected, proceeded through methanolic extraction using Schoofs *et al.*, (1990) [33] method to perform Bradford assay, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis **(**SDS-PAGE), disc diffusion assay, agar-well diffusion assay, and Minimum Inhibitory Concentration (MIC). Comparative estimates of protein concentration of the hemolymph samples showed that infected group at 24 hr of infection (I-24) had higher protein concentration as compared to the control group. SDS-PAGE for molecular weight determination showed prominent bands between 17 and 20 kDa in both control and infected groups. Disc diffusion assay of antimicrobial test showed small zones of inhibition in all the samples when they were treated with both gram-positive bacteria, *Bacillus cereus* and gram-negative bacteria, *Klebsiella pneumonia*. However, for agar-well diffusion assay, the zone of inhibitions was formed by all the samples when treated with gram positive bacteria, *B. cereus*. The Minimum Inhibitory Concentration (MIC) value of the sample C-24 was found to be 2.5 mg/mL against *K pneumonia*, and for sample C-24 and I-48 were found to be 1.25 mg/ml against *B. cereus.*

Keywords: *Philosamia ricini*, antimicrobial peptides, hemolymph, protein, SDS-PAGE, MIC

Introduction

To induce an innate immunity response in diverse categories of animals, including insects, Antimicrobial peptides (AMPs) are a broad class of chemicals that are essential components. This essential component serves as a primary line of defense against pathogenic invasion. When the insects encounter any kind of infections through microorganism they tend to produce antimicrobial peptides in a large quantity. As the invertebrates like insects do not possess an adaptive immune system, there immune response is induce by innate immune response components like AMPs as a part of defense response against bacteria and fungi (Ratcliffe., 1985 **[30]**; Jiang *et al*. 2010 **[18]**; Ouellette and Selsted., 1996) [26]. From the giant silk moth, *Hyalophora cecropia*, AMPs were first purified and isolated, in the year 1980. Till date, largely the AMPs have been known from the Lepidopteran insects which are cationic in nature and have amino acids lesser than 100 (Gao and Zhu., 2014) [13]. These insect peptides have an antibacterial property which ruptures the microbial membrane (Toke., 2005 **[36]**; Dai *et al.*, 2008 [9]; Huang *et al.*, 2008) [14] and also have the property to prohibit the development of drug resistance in microorganisms. AMPs are therefore regarded as an excellent option to replace antibiotics in clinical applications.

Upon bacterial infection in insects, a complicated genetic cascade is triggered, resulting in creation of a sequence of antimicrobial peptides that are let out into the hemolymph. This process gives insects their remarkable resistance to bacterial infections (Eleftherianos *et al.*, 2006 [11]; Eleftherianos *et al.*, 2007) [12]. Lipopolysaccharide (LPS)-binding protein, which is an inducible antibacterial peptide, was obtained from the hemolymph of an American cockroach, *Periplaneta Americana*. According to Hultmark (1993) [16], there are five main categories of antibacterial peptides are: cecropins, insect defensins, attacin-like proteins (glycine-rich), proline rich peptides and lysozymes. Eri silkworm, which is native to Northeastern states of India, and other regions of Southeast Asia, is highly prized for producing silk of the highest qualities. Nevertheless, eri silkworm provides important insights into the investigation of antimicrobial peptides and their possible uses in addition to its silk-producing skills. In the current investigation, the antimicrobial proteins from both infected and control hemolymph of the eri silkworm, *Philosamia ricini* was extracted which might insight for possible future research finding novel antimicrobial peptides.

Materials and methodology

Silkworm rearing

The eggs of silkworm *P. ricini* were pocured from Central Silk Board, Topatoli, Assam. To obtain the fifth instar larvae, the rearing was performed in the Zoology laboratory, Asssam Don Bosco University, on a diet of *Ricinus communis* leaves at the room temperature.

Immunization of 5th instars *P. ricini* larvae

After obtaining the 5th instar larvae, the samples were segregated into two groups such as, Control samples and another as Infected. To infect the samples larvae, it was injected with 10 µL of the attenuated *Escherichia coli* bacteria between the prolegs of the larvae using the Hamilton Microsyringe, while, the control samples were debarred of bacterial infections and only Phosphate Buffered Saline (PBS) was injected. The bacterial inoculum prior to the injection was prepared by inoculating it in the nutrient broth medium and incubating at 37°C for 24 hours and the bacterial cell adjusted to 0.5 McFarland i.e., 1.5 X 10^8 CFU/mL. To obtain the attenuated bacterial cells, the live bacterial cells were heat killed by keeping it in the hot air oven at 65°C for 1 hour (Sumida *et al.*, 1992) [35]. The infection period of the larvae *E. coli* was kept for 24 -48 hours and later processed for hemolymph collection.

Collection of hemolymph

Hemolymph was collected from the silkworms once after the completion of 24 hour of infections, and another after 48 hours, by cutting the tips of their pro-legs with sterile scissors. Later stored in a chilled falcon tubes containing a small amount of Phenylthiourea (PTU) to prevent oxidation of the collected hemolymph. The samples were then given codes as C-24, C-48, I-24 and I-48, where C-denoted the control samples collected at 24 and 48 hours of interval, and I-denoted infected samples collected at 24 and 48 hours. To obtain cell-free hemolymph samples, both the infected and control hemolymph were centrifuged at 5000 rpm for 5 minutes.

Extraction of proteins from crude hemolymph

The proteins from the collected hemolymph samples were extracted using the methodology mentioned in the Schoofs *et al.*, (1990) [33] with some modifications. Extraction buffer was prepared using methanol, glacial acetic acid and distilled water at the ratio of 90:1:9 (v/v). The buffer was then mixed with cell-free hemolymph samples, and it was incubated for 10 minutes before centrifuging for 30 minutes at 10,000 rpm at 4°C. The supernatant obtained was collected and methanol was evaporated. The remaining solution was freeze dried and obtained pellets were dissolved with 0.1% Trifluroacetic acid (TFA). For removal of lipids, n- Hexane was added into the solution and centrifuged at 20,000 rpm at 4°C, followed by freeze drying the samples. The pellets obtained were dissolved in the Phosphate Buffered Saline (PBS) solution and stored at -20°C for further study.

Protein estimation

Using Bradford method (Bradford., 1976) [23], the concentration of the extracted protein from the hemolymph was determined. Coomassie brilliant blue (CBB) dye was prepared by adding Ortho-Phosphoric acid (85%), ethanol and distilled water. The protein and the distilled water were taken at the ratio of 1:1 to obtain a total volume of 100 µl. 5 mL of CBB dye were added, and its Optical Density (OD) was measured at 595nm using the spectrophotometer. Bovine Serum Albumin (BSA) was use as a standard protein.

Statistical analysis

Completely Randomized Design (CRD) was employed to determine the results of the test for ANOVA (Analysis of Variance) to identify the significant difference of the protein content in the hemolymph samples using the Web-Based Statistical Software Package (WASP 2.0) Web Agri Stat Package of ICAR (Indian Council of Agricultural Research), Goa. Means were subjected to Duncan multiple range test to reveal Critical Difference (CD) that determines statistical significances.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed employing the methodology mentioned in the Lamelli (1970) [20], using 12% resolving gel and 5% stacking gel. Prepared hemolymph protein samples were place into separate wells corresponding to each lane- lane 1 with C-24, lane 2 with C-48, lane 3 with I-24, and lane 4 loaded with I-48. Prestained protein ladder with molecular weight in the range of 245-11kDa was also loaded into the gel to determine the molecular weight of loaded protein samples. Electrophoresis (Bio-Rad) was carried out at 100V for 3 hours. After electrophoresis, the gel was carefully removed from the cast and soaked overnight in the prepared staining solution followed by de-staining solution for 2-4 hours.

Antimicrobial activity test

Antimicrobial activity test was performed using the gram-positive bacteria- *Bacillus cereus* and gram-negative bacteria- *Klebsiella pneumonia*. These two bacteria were obtained from the Department of Biosciences, Assam Don Bosco University. For antimicrobial activity test, disc diffusion method (Hultmark *et al.*, 1983) [15] and agar-well diffusion method (Perez *et al.*, 1990) [29] was performed.

Disc diffusion assay

For this assay, bacterial cells were cultured in a nutrient broth medium for 24 hours at 37°C and its concentration of 0.5 McFarland i.e., 1.5×10^8 CFU/mL were measured using UV- visible spectroscopy at 600nm. The agar plates were prepared with the nutrient agar as its medium. Using a micropipette, to it 100µl of bacteria cells was introduces into the prepared plates, and employing the spread plate method, the bacteria were spread across the plate using the spreader. The sterile disc were then dipped in each eppendorf containing extracted proteins from the hemolymph samples, C-24, C-48, I-24 and I-48 with the sterile forcep and placed it on to a prepared plate. The antibiotic, Ampicillin was used as a standard for the current antibiotic assay. Plates were sealed properly with parafilm and incubated at 37°C for 24 hours.

Agar-well diffusion assay

This test was conducted by piercing a well on a prepared agar plate, and 50 µl of the samples (C-24, C-48, I-24 and I-48) were loaded in each well against the gram-positive and gram-negative bacteria. Afterwards, plates were incubated for 24 hours at 37°C and Ampicillin was used as the standard antibiotic.

Determination of Minimum inhibitory concentration (MIC)

MIC was determined using resazurin microtiter assay as per Borkotoky and Borah (2023) [3], with some modifications. For this test, the same concentration of gram-positive and gram-negative bacteria was adjusted to 0.5 McFarland turbidity standards (1.5×10^8 CFU/mL). Samples were loaded into a 96-well microtiter plate applying the method of serial dilution. The plate was sealed with a parafilm and incubated at 37°C for 24 hours. After 24 hours of incubation, 0.02 % resazurin dye (Himedia-RM 125-1G) was added to each well and incubated for 1 hour at 37°C. The transition of color from blue to pink was observed. Wells having microbial growth changed its color from blue to pink, indicating growth of the bacteria, whereas, the wells that did not had any microbial growth remained blue in its color. Ampicillin was used as positive control, whereas, for negative control, the broth and bacteria were used.

Results

Protein estimation

Estimation of protein content employing Bradford assay for control and infected group of Eri silkworm, *P. ricini* as showed that the protein content was highest in 24 hours of infection (I-24) with a total protein content of 2.42 mg/mL, and lowest in the sample I-48 with a value of 2.22 mg/mL (Fig 1 and Table 1). The samples C-24 and C-48 that were control samples possessed a total protein concentration of 2.31 mg/mL, and 2.23 mg/mL respectively. Through ANOVA (Analysis of Variance) test, it was found that Coefficient of variation (CV) was 2.574 and Critical difference (CD) at 0.05 was found to be 0.091, indicating the treatment as significant.

Table 1: Estimation of protein content from the infected and control hemolymph of Eri silkworm, *P. ricini* using Bradford method

Sample name	OD at 595 nm	Protein concentration (mg/mL) ± SD
C 24	0.247	2.31^{b} ± 0.014
C 48	0.233	2.23^{b} ± 0.011
I 24	0.267	2.42^{a} ± 0.012
I 48	0.231	2.22^{b} ± 0.002
CV= 2.574 CD (0.05)= 0.091		

*Means followed by same letter(s) as superscript in the same column are not significantly different ($p<0.05$) using Duncan Multiple Range Test (DMRI).

*CV = coefficient of variation; CD = critical difference; SD = Standard Deviation.

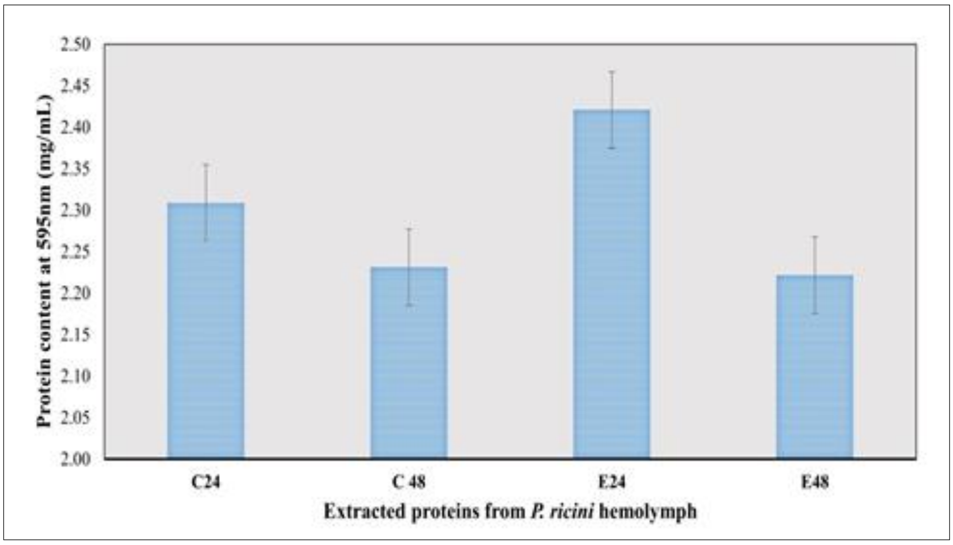

Fig 1: A bar chart showing total protein content of the control and infected hemolymph of *P. ricini*

SDS-PAGE analysis

After staining and de-staining, the gel, a protein band with molecular weight of 75kDa band was observed in all the samples, with greater intensity in lane-1 (C-24 sample) (Fig 2**)**. Additionally, 63kDa band was observed in C-24, but was not observed in the other samples. Furthermore, a thin line of band between 63-48kDa was seen in all the samples (C-24, C-48, I-24 and I-48). Another thin band were also observed in lane-1 (C-24) and lane-4 (I-48) between 17-20kDa.

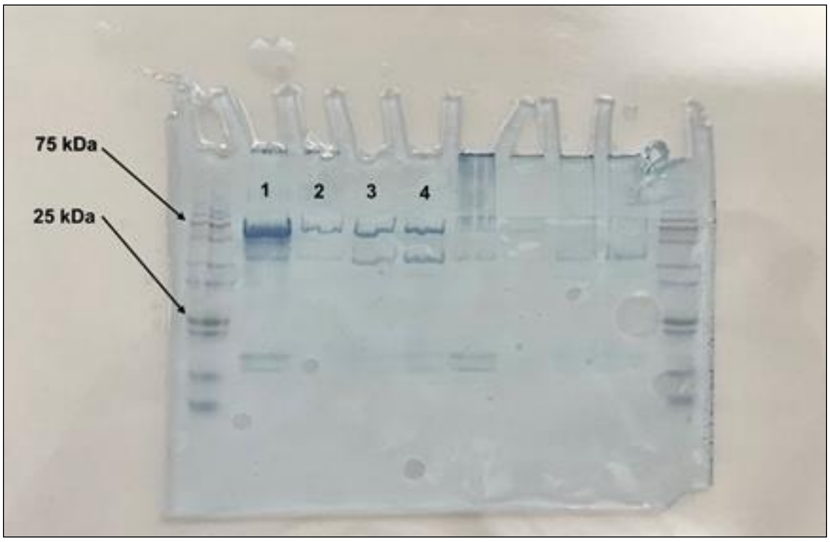

Fig 2: SDS-PAGE analysis of extracted protein from hemolymph of *P. ricini*

Antimicrobial-activity test

Disc diffusion assay

The antimicrobial activity test of C-24, C-48 (control samples) I-24 and I-48 (infected sample) were performed against two bacterial strains, *Klebsiella pneumonia* (gram-negative) and *Bacillus cereus* (gram-positive) as shown in the Fig 3. After incubating the plate for 24hr, zones of inhibitions was measured and noted as displayed in Table 2**.** It was recorded that for gram-negative bacteria *K. pneumonia*, the mean diameter zone of inhibition formed around the disc for the sample C-24 was 6.5 mm, C-48 was 6.5 mm, I-24 was 6.5 mm and for I-48 was 7.0 mm respectively. However, for gram-positive bacteria *B. cereus*, C-24 showed 7 mm zone of inhibition, C-48 as 6.5 mm, I-24 as 6.5 mm, and I-48 as 6.5 mm respectively. Here, with the gram-positive bacteria the findings showed highest in the control samples and lesser in the infected samples. The zone of inhibition for Ampicillin was 8mm.

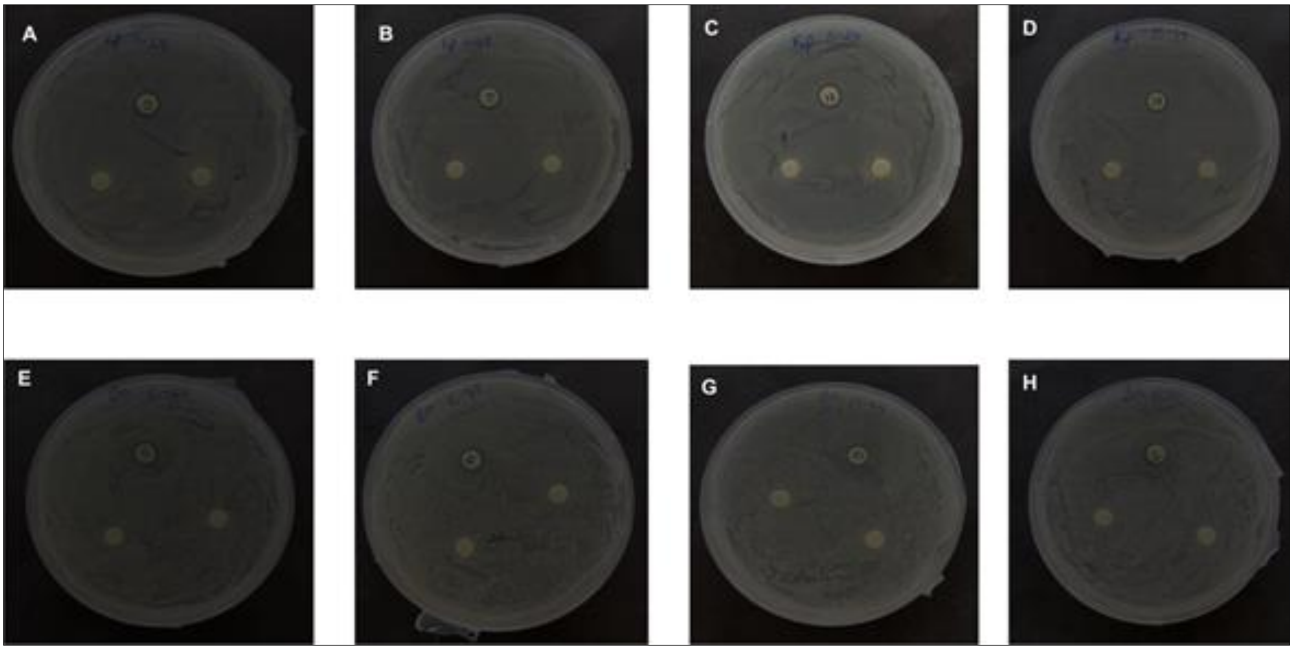

Fig 4: Antimicrobial activity test of the extracted protein from hemolymph of *P. ricini* against gram-positive, *Bacillus cereus* (A, B, C, D) and gram-negative bacteria, *Klebsiella pneumonia* (E, F, G, H) by well-diffussion assay

A C-24. **B** C-48. **C** I-24. **D** I-48. **E** C-24. **F** C-48. **G** I-48. **H** I-24

Table 2: Antimicrobial activity test and MIC of the proteins extracted from eri silkworm's hemolymph *P. ricini* against *B. cereus* and *K. pneumonia*

Sl. no	Sample	Antimicrobial activity test (mm) Disc diffusion assay		Antimicrobial activity test (mm) Well diffusion assay		Minimum inhibitory concentration (mg/mL)	
		B. cereus	*K. pneumonia*	*B. cereus*	*K. pneumonia*	*B. cereus*	*K. pneumonia*
1	C-24	7 .0	6.5	Mild	6.5	1.25	2.5
2	C-48	6.5	6.5	Mild	6.5	Mild	Absent
3	I-24	6.5	6.5	Mild	7.0	Mild	Absent
4	I-48	6.5	7.0	Mild	6.5	1.25	Absent

Agar-well diffusion assay

Similarly, the same concentration of *K. pneumonia* and *B. cereus* were utilized for well diffusion assay against the samples C-24, C-48, I-24 and I-48. The test results are shown in Fig 4. A distinct zone of inhibition was seen in samples C-24 (6.5 mm), C-48 (6.5 mm), I-24 (7 mm) and I-48 (6.5 mm) against *B. cereus* after 24 hours of its incubation, as shown in Table 2, whereas the samples C-24, C-48, I-24 and I-48 against the gram-negative bacteria, *K. pneumonia*, inhibition zone was not visible. Zone of inhibition for Ampicillin was 25 mm.

Minimum inhibitory concentration

After loading the sample, C-24, C-48, I-24 and I-48 in a 96-well micro titer plate by serial dilution, Resazurin dye was put into each well to examine the transition of color from blue to pink of the infected (I-24 and I-48) and control (C-24 and C-48) samples against the *B. cereus* and *K. pneumonia*. Fig 5 shows the change in color from blue to pink indicating the bacterial resistance towards the sample, and its tendency to grow against the protein sample.

In the examined samples, it was recorded that C-24, C-48, I-24, and I-48 against *K. pneumonia* retained blue color in 10 mg/mL concentration, indicating a positive antimicrobial property (Fig 5A). However, in the second row of the test plate where the concentration of the sample was 5 mg/mL, the positivity was shown in sample C-24 and I-48, while rest of the samples did not exhibit antimicrobial property. While the lower concentration of sample, failed to express its MIC. The inhibitory test against the bacteria, *B. cereus*, using the samples expressed a better inhibitory property even in the lower dilutions (Fig 5B). The inhibitory property of the samples was visible in 10 mg/mL, 5 mg/mL, 2.5 mg/mL and 1.25 mg/mL of the concentrations in the entire sample (C-24, C-48, I-24 and I-48) against *B. cereus*, indicating the effectiveness of samples even in lower concentration.

The lowest MIC value against *K. pneumonia* was 2.5 mg/mL that was observed in C-24 sample, whereas, for *B. cereus*, the lowest MIC value was found to be 1.25 mg/mL in C-24 and I-48 samples (Table 2). However, mild retention of blue color was observed in C-48 and I-24 samples at the concentration of 1.25 mg/mL indicating the presence of antimicrobial property.

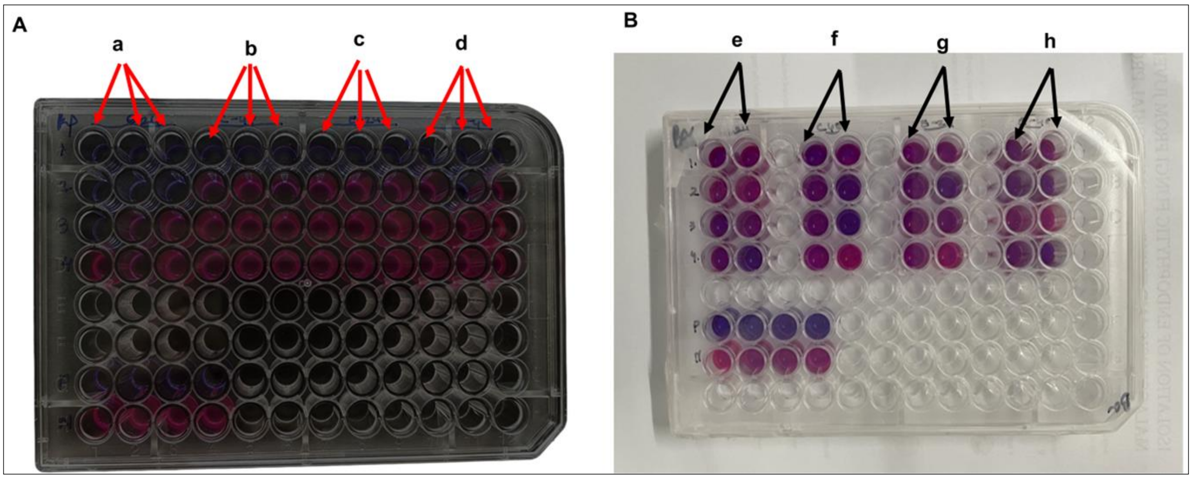

Fig 5: Minimum inhibitory concentration of the purified protein from hemolymph of *P. ricini* against (A) gram-negative, *Klebsiella pneumonia* and (B) gram-positive, *Bacillus cereus*
A C-24. B C-48. C I-24. D I-48. E C-24. F C-48. G I-24. H I-48

Discussion

Antimicrobial peptides, a crucial component of innate immunity exhibits a significant role against invading microbes (Brey and Hultmark., 1998) [4]. Numerous systems in insects cooperate to prevent the spread of bacteria and other diseases. Their bodies are feature with pattern-recognition proteins, which allow them to synthesize antibacterial peptides that can adhere to the external surface of bacteria or other pathogens (Vilcinskas., 2013) [37]. AMPs have the ability to physically damage the cellular membranes of pathogenic microbes leading to their death. The microbial membrane is therefore typically regarded as the major targets of AMPs (Chernysh *et al.*, 2015 [7]; Shen *et al.*, 2018) [34]. A number of methods such as membrane disruptions, disruptions of bacterial metabolism, and targeting of cytoplasmic components, are employed by AMPs to cause the death of bacteria (Ongey *et al.*, 2018 [25]; Patocka *et al.*, 2018 [28]; Shen *et al.*, 2018 [34]; Jozefial and Engberg., 2017 [19]; Bechinger and Gorr., 2017 [2]; Onate-Garzon *et al.*, 2017) [24].

In this study, the hemolymph proteins of *P. ricini*, and their antibacterial activity against both gram-negative and gram-positive bacterial strains were examined. The control sample (C-24 and C-48) in the disc diffusion assay exhibited minimal antimicrobial activity against both gram-positive and gram-negative bacteria; similarly, the infected hemolymph (I-24 and I-48) also expressed a similar type of antimicrobial property against the bacterial strains. This phenomenon indicates the existence of immune response in the *P. ricini* even prior to the artificial ways of immunity induction which they might have naturally acquired from its progenitor. In well diffusion assay, both infected and control samples exhibited inhibitory actions against *B. cereus*, which was gram-positive bacteria. However, both the control (C-24, C-48) and the infected (I-24, and I-48) samples did not exhibit any inhibitory action against the gram-negative bacterial strain *K. pneumonia*. MIC was determined to assist the development of antibiotic drug resistance (Wiegand *et al.*, 2008) [38]. The samples in this study were discovered to be very much effective in exhibiting its antimicrobial property against the gram-positive type of bacteria *B. cereus*, even at the lower concentration. However, the samples effectiveness against the bacteria *K. pneumoni,* which was a gram-negative type, was comparatively lower. These conditions indicate the variations of the antimicrobial property of the hemolymph against certain bacteria, where some bacteria try to express a defensive property, while some bacteria are easily prone to it. Also, the reason for exhibiting a higher antimicrobial property against the gram-positive bacteria and lesser against the gram-negative type could be due to the structural differences. Gram-negative bacteria are bounded by an outer membrane which forms the key structural differences with the gram-positive type (Breijyeh *et al.*, 2020) [5]. This outer membrane is composed of phospholipids and is the chief reason for the structural integrity which shows or functions as a protective layer against certain antibiotics. Such defensive structural component is lacking in the gram-positive type of bacteria which makes them prone to the antimicrobial agents. This could be the possible reason why in the present study the protein samples antimicrobial property expressed a higher inhibitory activity against the gram-positive type of bacteria and lesser against the gram-negative type. Therefore, at certain conditions when the peptides fail to express inhibition property or expressed lesser inhibition property it does not mean that antimicrobial peptides are absent; rather, it might imply that they are present in smaller amounts and do not exhibit any significant activity *in vitro* might depend on the specificity of certain bacteria. Similar observations were reported by Basseri *et al.*, (2016) [1]. It has been found that in reaction to foreign invaders or non-self-recognition, various insect species may produce different antimicrobial peptides (Engstrom., 1999) [10]. Metchnikowins peptides, which was separated from *D. melanogaster*, failed to show activity against gram-negative bacteria, but effectively prevented the bacteria like *Micrococcus luteus* from growing which was a gram-positive type (ImLer and Bulet., 2005 [17];

Rahnamaeian *et al.*, 2009 [31]; Rahnamaeian and Vilcinskas., 2012) [32]. Also, when the extracted proteins were run through the SDS-PAGE, 17-20 kDa were visible in C-24 and I-48 hemolymph samples, indicating the presence of antimicrobial proteins. According to Buhroo *et al.*, (2018) [6], AMPs have been reported to contain low molecular weight proteins that are smaller than 30 kDa, or less than 100 amino acids.

When bacteria are introduced into the bodies of insects, a number of peptides and proteins are produced as part of the immunological response. These substances have the ability to work alone or in cooperation to combat the foreign invaders (Cocianchi *et al.*, 1994 [8]; Vilcinskas., 2013) [37]. Furthermore, it is possible that the proteins found in this study may be crucial to *P. ricini*, individually or collectively defending against bacterial infections. However, further research is required in this regard. It is important to remember at this point how certain infectious bacteria might develop resistance to basic antibiotics such as gentamicin, penicillin etc. Antimicrobial peptides are also expected to be used in the near future as alternatives to antibiotics (Malgorzata *et al.*, 2007) [21]. The rewards of antimicrobial peptides are variety, including, ability to eliminate bacteria quickly, broadly and low resistivity (Matsuzakai., 1999 [22]; Papo and Shai., 2005) [27].

Conclusion

The main objective of this research was to separate the antimicrobial proteins from the hemolymph of Eri silkworm, *P. ricini* and examine their efficacy against gram-positive and gram-negative bacteria. It was noted that, when an immunological challenge is present, *P. ricini* induced hemolymph can synthesize AMPs against gram-positive bacteria. The effectiveness of the extracted proteins exhibited higher antimicrobial property against the gram-positive type and minimal activity with gram-negative type, owing to a structural dissimilarities in the current study. In future, to enable the peptides to function on pace with or better than conventional antibiotics, more work are required to enhance the purification processes of antibacterial proteins without causing damage to the proteins. Also, sequencing the proteins would also be beneficial for future studies to identify the specific peptides which might be responsible for inducing antimicrobial property against certain microbes.

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Declarations of competing interest

The authors declare no competing of interest.

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