Induction of antimicrobial peptides from *Bacillus thuringiensis* challenged *Spodoptera litura* larvae and investigation of the antimicrobial properties of hemolymph samples

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Abstract

Antimicrobial peptides constitute key factors in insect humoral immune response against invading microorganisms. In this study, biochemical approach was identified antimicrobial peptides which appeared in larval hemolymph of Spodoptera litura after bacterial challenge. HPLC profile showed two major peaks in two samples, Brassica oleracea and Ricinus communis fed S. litura that were collected at 5 min interval. It was shown to be active against Gram-positive and Gram-negative bacteria. The highest zone of inhibition was observed in Staphylococcus aureus and Escherichia coli in B. oleracea fed S. litura hemolymph fraction II and R. communis fed S. litura hemolymph fraction I and it also contributes the increased antioxidant, lysozyme, and less hemolytic activity were increase in treated groups. TLC activity was tested with hemolymph extract samples, pink color pots was identified the protein present in the samples. An SDS-PAGE result shows that high expression of antimicrobial peptide present in the treated sample. The appearance of peptides with such different properties in insect hemolymph in response to immune challenge indicates the complexity of the insect immune system.

Keywords: Insect immunity; *Spodoptera litura*; *Bacillus thuringiensis*; Lysozyme; Antioxidant; Antimicrobial peptide

1. Introduction

Insects are the most successful, largest group in worldwide (Saito et al., 2004). Insects develop the resistance mechanisms against the pesticide and microbial pathogens. This may develop the immunity through the cell mediate and humoral mediated immune response (Lavine and Strand 2001, 2003). A cellular immune response including hemocyte aggregation and nodulation, encapsulation, and changes in hemocyte levels (Ribeiro and Brehelin, 2006) and humoral defense mechanisms production of antimicrobial peptide (Lavine and Strand 2002).

Antimicrobial peptide (AMP) is an important defense mechanism against the pathogen. It produced mainly from tissues like fatbody, and secreted into the hemolymph. AMP ribosomally synthesized polypeptide, having 30-60 or less than 100 amino acid residues. Most AMP acts against gram-positive and gram-negative bacteria, fungi, viruses, protozoa, and nematodes (Sarkar et al., 2021). AMPs are classified into five groups, namely cecropin, defensin, glycine-rich peptide, proline-rich peptide, and lysozyme (Bulet et al., 1999). Cecropin was first isolated from *Hyalophora cecropia*, against bacteria and fungal infection (Steiner et al., 1981). Cecropins has been isolated from several lepidopteran and dipterans insects. Defensin peptide is another AMP which produces strong activity against gram-positive (Hetru et al., 2003).

Lepidopteran insect model organisms used for studying insect immune response, and also pathogenicity tests against several microbes. In order lepidoptera, *Spodoptera litura* is a polyphagous insect which causes lot of economic loss in crop fields (Pogue, 2002). This insect mostly controlled by chemical insecticides, during decades leads to cause the problem. So in public health concern to investigating the safety and alternative methods for controls the insects (Andreev et al. 2008). Recently microbes and their metabolites are used as cheap and safety methods for controlling the insects. *Bacillus thuringiensis* (Bt) strains and their metabolites are mostly used to control lepidopteran larvae (Ferre and Van Rie, 2002). These insect pest have developed several resistance mechanisms against Bt toxins (Zhu et al., 2016)

Larval immune hemolymph has main defense mechanisms for the production of antimicrobial peptides when challenged with microbes. In these study the better knowledge of the diversity of AMP production against the microbial infections and also to determine the specific antimicrobial activity against several microbes. The purpose of the present study is aimed at the humoral defense mechanisms and antimicrobial activity of hemolymph peptides from *Bt* challenged *S. litura*.

Materials and Methods

Treatment of larvae with Bt 4D1 and preparation of hemocyte-free hemolymph

S. litura larvae were maintained on *B. oleracea* and *R. communis* for upto 3 generations has reported in our previous study (Vengateswari et al., 2020). Here the host plant maintained larvae were used for this study and larvae were immunized with an injection of Bt 4D1 cells and kept in dark at 24 h. After 24 h the insects were chilled for 10 min at 4^{o}C using a freezer then the surface was sterilized using 75% of ethanol. Hemolymph samples were collected by puncturing of larval abdomen leg with the use of a sterile needle. Then the collected hemolymph sample was immediately transferred into effendorf tubes containing a

phenylthiourea (PTU) (Cytorynska et al., 2007). Then the hemocyte-free hemolymph samples were centrifuged at 1800 rpm for 15 min and then the cell-free hemolymph sample (1000µl), and stored at -80 °C for a further investigation. The same procedure was applied to the control group with an injection of saline.

Preparation of hemolymph extracts

The extraction methods were followed by Schoofs et al. (1990). 90% methanol, 1% glacial acetic acid, and 9% of water were used for cell-free hemolymph extraction. The solvent mixed samples were centrifuged at 14,000 rpm for 30 min. The supernatant was collected and evaporated through the rotary evaporator. Then the precipitated proteins were collected and freeze-dried with adding 0.1% trifluoroacetic acid (TFA). Adding of n-hexane for removal of lipid content from the sample and the sample was vortexed and centrifuged at 14000 rpm for 10 min at 4°C. Lipid containing the upper layer was removed and add an equal volume of ethyl acetate for a water-soluble fraction (containing peptides). After centrifugation, the soluble fraction was freeze-dried and stored at -80 °C for further uses.

RP-HPLC analysis

Hemolymph extract (*B. oleracea* and *R. communis* fed *S.litura* larval hemolymph) was dissolved in 0.1% TFA for purification by reverse –phase HPLC, LC-18 (4.6 mm x 250mm) column. Two buffer sets were used, A: 0.1% TFA (v/v), B: 0.07% TFA in 80% acetonitrile. A linear gradient from 5-70% of buffer B was used to collect fractions over 50 min periods with a flow rate of 2.5 ml/min monitored at 215 nm wavelength. The fractions were collected manually at 5 min interval and the collected fractions were again purified two times. Each fraction was collected separately, freeze-dried, resuspended in 1x phosphate-buffered saline (PBS) and antimicrobial activity was tested.

Antimicrobial activity assay

The presence of antibacterial activity in the purified hemolymph was detected by a growth inhibition zone assay using solid agar plates containing viable microbes as described by Hoffmann et al. (1981). The hemolymph extract was tested against the following pathogenic microorganisms, *Escherichia coli, Pseudomonas aeruginosa, serratia marcescens, Bacillus subtilis,* and *Pseudomonas fluorescens* (Gram-negative bacteria), *Bacillus thuringiensis* and *Staphylococcus aureus*, *Enterococcus faecalis, Micrococcus luteus,* and *Bacillus cereus* (Gram-positive bacteria). The collected fractions was dissolved in PBS and three concentrations was prepared (12.5, 25, 50 µg/ml) were added into the well. The agar plates were incubated at 37 °C for 24 hours. The diameters of growth inhibition zones were measurements followed by Hultmark et al. (1982).

Total antioxidant properties of purified peptide

0.1 ml of the hemolymph extract sample was taken in a vial and 0.5 ml of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was added. Sample mixture was incubated for 30 min in a dark. After the incubation, the antioxidant scavenging activity was measured by using the spectrophotometer at 620 nm.

Lysozyme activity

Lysozyme activity of purified hemolymph extract was determined by the method of Binuramesh and Michael (2011). *Micrococcus luteus* bacterial suspension was dissolving with 0.04 M potassium phosphate buffer with pH 7.2 and OD at 520 nm. Firstly, 175 µl of the bacterial suspension was put into one well of a 96-well culture plate, and then 25 µl purified samples were mixed by vortex, and the OD was recorded at 520 nm.

Hemolytic activity

Hemolytic activity was performed using goat erythrocytes as followed by Ryge and Hansen, (2004) with slight modification. Collected blood samples were washed 3 times (3000rpm) with a cold solution of 0.15M, pH 7.2 PBS and erythrocytes were diluted to a final concentration of 0.5% in PBS. To each well of a polypropylene microtiter plate was added 75 µl of diluted erythrocytes and 75 µl of peptide solution at different concentration 50, 25, 12.5 µg/ml was added. The microtiter plate was incubating at 37^{o}C for 1h. The samples were centrifuged for 10 min at 4000 rpm and 100 µl of the supernatant was transferred into new 96-well plates. The absorbance was recorded at 414 nm on ELISA plate reader. Suspending the erythrocytes in 10% of PBS and 0.1% of triton X 100 was used as a reference, negative control (zero hemolysis) and positive control (100% hemolysis). The percentage of hemolysis was calculated as follows: $[(A_{peptide}-A_{PBS}) / (A_{Triton}-A_{PBS})] \times 100$. The experiment performed in triplicate and are the average of three independent determinations using the same stock solution.

Thin Layer Chromatography (TLC)

The purified peptide was further analyzed by thin-layer chromatography to check any contaminants present in the purified sample. The TLC chamber was saturated with the solvent, butanol, acetic acid, and water in the ratio of 4:1:5. The 10 µl of peptide sample or glycine (as standard) was spotted in the silica-coated TLC plate above the 1cm line from the bottom. The spots were allowed to dry, after drying the TLC plate was placed in the chamber equilibrated with solvent the plate such that the spots stayed above the solvent. When the solvent drowns by capillary action reached almost another end of the plate. Then the TLC plates were taken and the solvent front was marked approximately 1cm from the top. The

slides were air-dried and sprayed with 0.2% ninhydrin, for development of pink-colored spots, the plate was dried in a hot air oven at 65^{o}C for 15 min and photographed (Ravichandran et al., 2010). Rf value was calculated for well using the following formula:

Rf =Distance traveled by solute/ Distance traveled by the solvent.

SDS-PAGE Analysis

To estimate protein expressed in the hemolymph purified sample was performed by Sodium Dodecyl Sulphate Polyacrylamide Gradient Gel Electrophoresis (SDS-PAGE) buffer system. 10 µl of purified hemolymph sample was diluted with adding 50 µl of ice-cold, 4% SDS containing Tris HCl buffer with EDTA-free protease inhibitor cocktail (Pierce) and directly stored at -20°C. For measurements, stored samples were allowed centrifuged at 9200 g for 15 min. The collected supernatant was transferred to new tubes and add the loading dye to the supernatant and the sample heated in boiling water for 5-10 min. Prepared samples were loaded into 15% of SDS-PAGE on each well. The electrophoresis was carried out at a constant 50 volts using a mini protean III electrophoresis cell. After electrophoresis, the gel was stained with a staining solution (200 mg of Coomassie brilliant blue added with 7 ml of methanol and dissolved with 43 ml of distilled H_2O). Again gel was destained with a 30:7 ratio of methanol: acidic acid solution. The molecular weight of the proteins was determined using a standard protein molecular weight marker.

Determination of minimum inhibitory concentration

Mid log phase culture of *S. aureus* and *E. coli* was diluted to approximately 10^5 cells/ml in LB medium. The purified peptide was serially diluted in a twofold manner and 10 µl of these diluted peptides were added to the 96-wells plate followed with the addition of 90 µl of the bacterial suspension. The plate was incubated for 24 h at 37 °C. After incubation, the OD value of each well was measured at different time (0, 2, 4, 6, 8, and 10 h) intervals at 600 nm in ELISA reader. Experiments were performed in triplicates for each concentration (20, 40, 60, 80 and 100 µg/ml) of peptide and the MIC value was determined following the guidelines of CLSI, 2007 (CLSI 2010). The growth curve of *S. aureus* and *E.coli* was also prepared in the presence and absence of purified peptide.

Statistical analysis

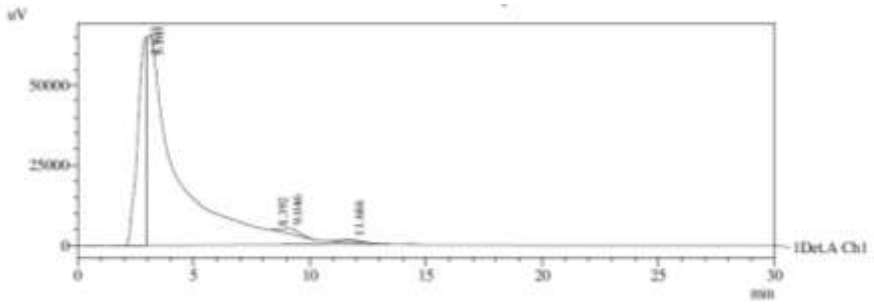
All experimental assays were performed using three replications, and the obtained assays data were analyzed by two-way ANOVA (Bonferroni post-test) using PRISM 5 software. The P-value (<0.05) was considered significant.

Results

Extraction and purification of AMP

The crude extraction was separated by reverse-phase HPLC, LC-18 (4.6 mm x 250mm) column. HPLC profile showed two major peaks in two samples that were collected at 5 min interval (Fig. 1). *B. oleracea* and *R. communis* fed *S. litura* larval hemolymph have two fractions from each samples. Fig.1 A and B showed *B. oleracea* and *R. communis* hemolymph has two major peaks were collected again purified by preparative column. The high peak was observed in the 2-3 min time intervals both samples.

a



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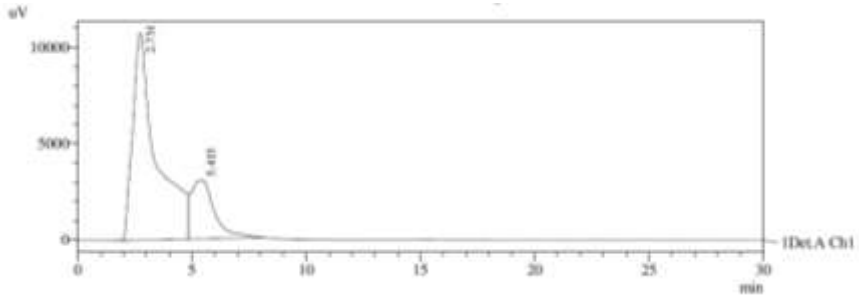


Figure 1: Purification of peptide from *S. litura* immune-hemolymph extract. Equivalent of 100 ml of hemolymph was separated on a C-18 column using water/TFA/acetonitrile buffers set. (a) *B. oleracea* hemolymph purified extract. b) *R. communis* fed hemolymph purified extract

Antimicrobial activity

The antimicrobial activity of the purified fractions against different Gram-positive and Gram-negative bacteria was examined. The obtained results are summarized in Table 1 and 2. Antimicrobial activities of the *B. oleracea* fed *S. litura* hemolymph

Tested microorganisms	Tetracycline (St)(µg/ml)			Crude (µg/ml)			Fraction I (µg/ml)			Fraction II (µg/ml)		
	50	25	12.5	50	25	12.5	50	25	12.5	50	25	12.5
S. aureus (+ve)	+++	+++	++	+++	++	+	++	++	+	+++	+++	++
B. thuringiensis (+ve)	+++	+++	++	++	+	+	++	+	+	+++	++	+
M. luteus (+ve)	+++	++	++	++	+	+	++	+	+	+++	++	+
E. faecalis (+ve)	+++	+++	++	+++	++	+	++	+	+	+++	++	+
B. cereus (+ve)	+++	+++	++	+++	+	+	++	++	+	+++	++	+
E. coli (-ve)	+++	+++	++	+++	++	0	+	0	0	+	0	0
P. aeruginosa (-ve)	+++	+++	++	++	+	0	+	+	0	+	0	0
S. marcescens (-ve)	+++	++	++	+	0	0	+	+	0	+	0	0
P. fluorescens (-ve)	+++	+++	++	++	+	0	+	0	0	+	0	0
B. subtilis (-ve)	+++	+++	++	+	+	0	+	0	0	+	0	0

Table 1 Antimicrobial activity of the extracted *S. litura* peptides against different gram-positive and gram-negative bacteria was examined. St: standard, 0: No inhibition of growth, +: inhibition value 5-10 mm +: inhibition value 11-15 mm, ++: inhibition value 16 -20, +++ mm

Antimicrobial activities of the *R. communis* fed *S. litura* hemolymph

Tested microorganisms	**Tetracycline (St)(µg/ml)**			**Crude (µg/ml)**			**Fraction I (µg/ml)**			**Fraction II (µg/ml)**		
	50	25	12.5	50	25	12.5	50	25	12.5	50	25	12.5
S. aureus (+ve)	+++	+++	++	+++	++	+	+++	+++	++	++	+	+
B. thuringiensis (+ve)	+++	+++	++	+++	+	+	+++	++	++	++	+	+
M. luteus (+ve)	+++	++	++	+++	+	+	+++	++	++	+++	++	+
E. faecalis (+ve)	+++	+++	++	+++	++	+	+++	++	+	++	+	+
B. cereus (+ve)	+++	+++	++	+++	+	+	+++	++	+	++	+	+
E. coli (-ve)	+++	+++	++	+++	+++	++	++	+	+	++	+	+
P. aeruginosa (-ve)	+++	+++	++	+++	+++	++	++	+	+	+	+	+
S. marcescens (-ve)	+++	++	++	+++	++	+	++	+	+	+	+	0
P. fluorescens (-ve)	+++	+++	++	+++	++	+	++	+	+	+	+	0
B. subtilis (-ve)	+++	+++	++	+++	++	+	++	+	+	+	+	0

Table 2 Antimicrobial activity of the extracted *S. litura* peptides against different gram-positive and gram-negative bacteria was examined. St: standard, 0: No inhibition of growth, +: inhibition value 5-10 mm +: inhibition value 11-15 mm, ++: inhibition value 16 -20, +++ mm

The highest inhibitory activity was observed in *B. oleracea* fed *S. litura* hemolymph fraction II and *R. communis* fed *S. litura* hemolymph fraction I when compared to the control and crude extract. High inhibition activity was observed in *S. aureus* and *E. coli* in both fractions. Among the bacteria examined, the Gram-positive bacteria were more sensitive than the Gram-negative bacteria (Fig 2).

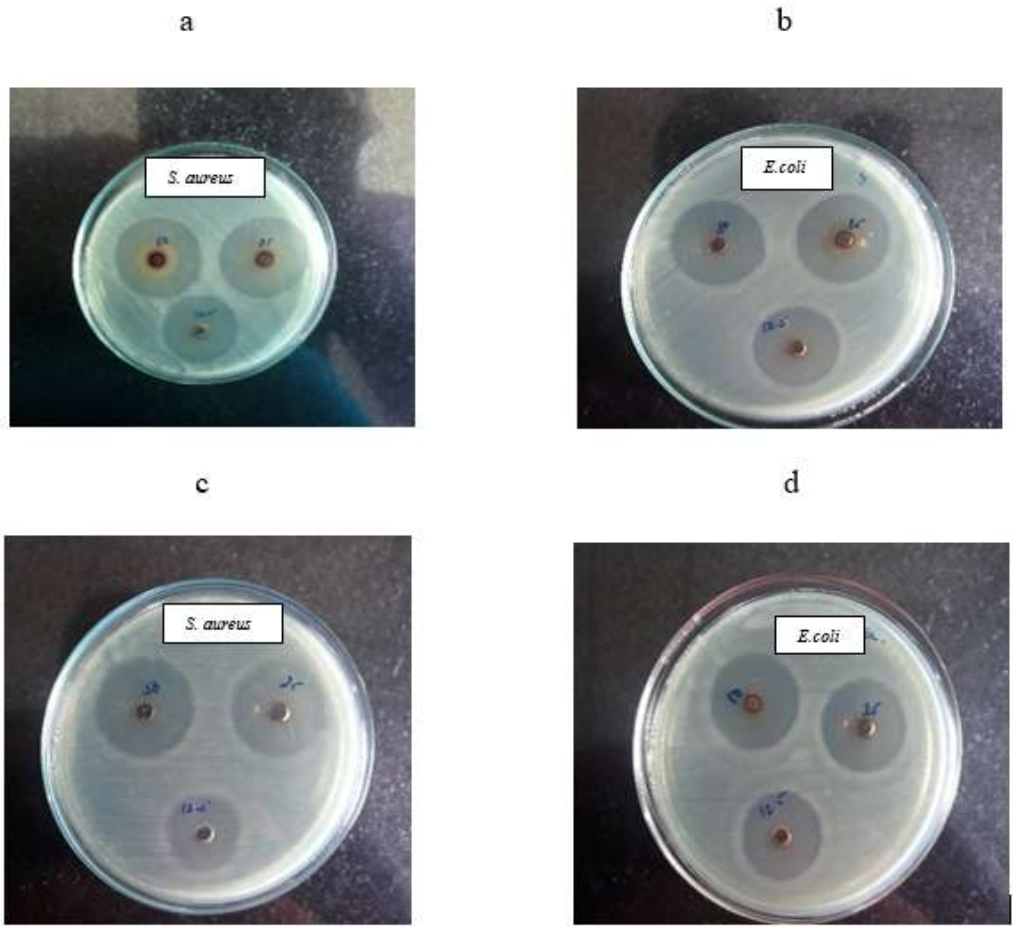


Figure 2: Antibacterial activity of *S.litura* purified fraction against gram positive bacteria and gram negative bacteria *S. aureus* and *E.coli* a) and b) *B. oleracea* fed *S. litura* purified hemolymph peptide, and c) and d) *R. communis* fed *S. litura* purified hemolymph peptide.

Total antioxidant activity

Total antioxidant capacity was examined in *B. oleracea* and *R. communis* fed *S. litura* hemolymph purified fractions. High level of antioxidant properties was observed in *R. communis* fed *S. litura* hemolymph fraction I and *B. oleracea* fed *S. litura* hemolymph fraction II when to compare to other fraction (Fig. 3)

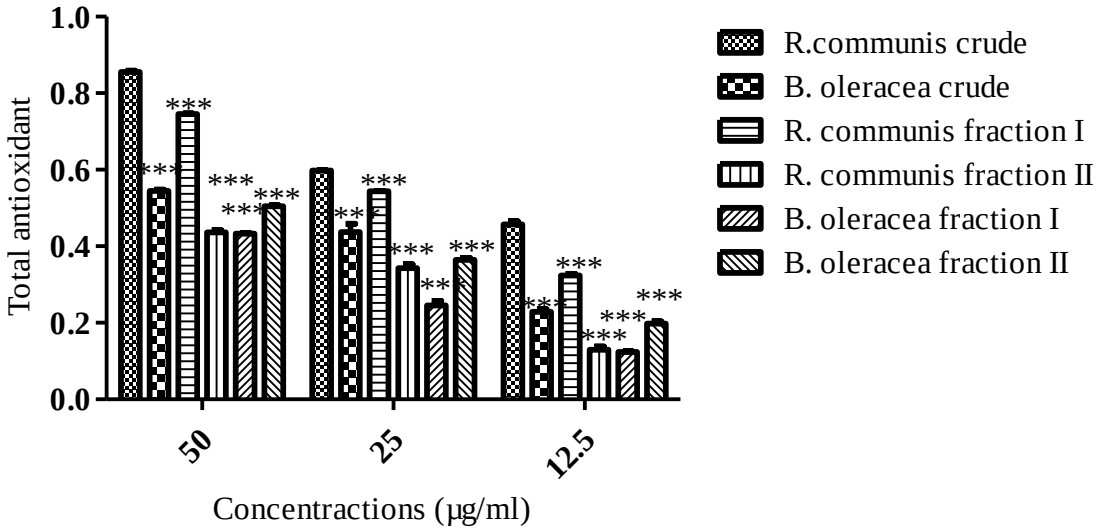


Figure 3: Total antioxidant properties of purified peptide from *S. litura* larvae. The values are expressed as mean (±S.D) analyzed by two- way ANOVA. Asterisk (*) indicates significant difference among treatments with respect to control. Note (***) indicates (p<0.0001).

Lysozyme activity

We investigated whether the level of lysozyme activity changed after treatment with *B. thuringiensis*. It is known that lysozyme plays an important role in the humoral defense of *S. litura*. High lysozyme activity observed in *R. communis* fed *S. litura* hemolymph fraction I and *B. oleracea* fed *S. litura* hemolymph fraction II was incubated with *M. luteus* (Fig 4).

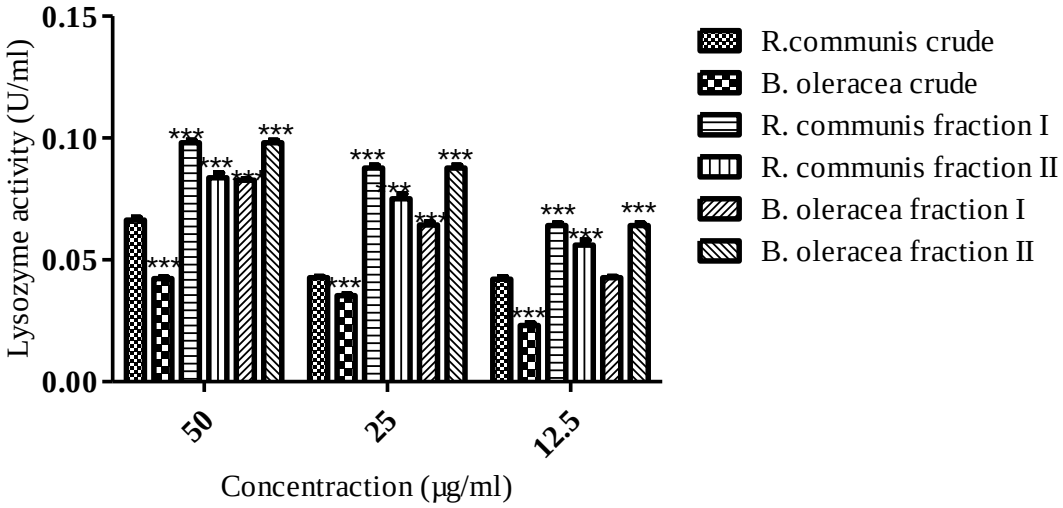


Figure 4: Lysozyme activity of purified peptide from *S. litura* larvae. The values are expressed as mean (±S.D) analyzed by two- way ANOVA. Asterisk (*) indicates significant difference among treatments with respect to control. Note (***) indicates (p<0.0001).

Hemolytic activity

The lytic activity was examined in goat erythrocytes with purified fractions. The *R. communis* fed *S. litura* hemolymph fraction I and *B. oleracea* fed *S. litura* hemolymph fraction II was weaker hemolytic activity than *R. communis* fed *S. litura* hemolymph fraction II and *B. oleracea* fed *S. litura* hemolymph fraction I. Highest percentage of hemolysis (0.02-0.09%) were observed at higher concentration of 25 and 50 µg/ml (Fig. 5).

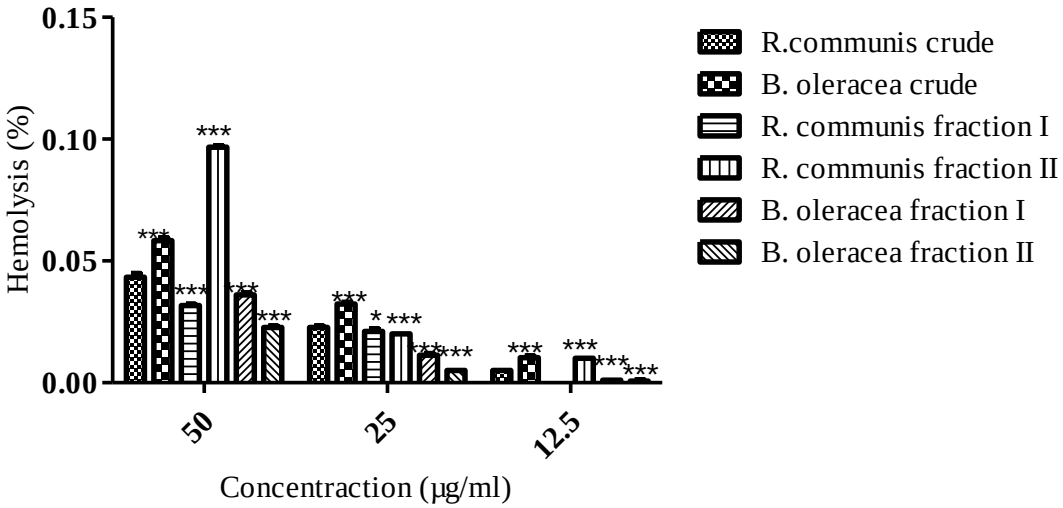


Figure 5: Hemolytic activity of purified peptide from *S. litura* larvae. The values are expressed as mean (±S.D) analyzed by two- way ANOVA. Asterisk (*) indicates significant difference among treatments with respect to control. Note (***) indicates (p<0.0001).

Protein Detection by using TLC

The purified peptide was analysis using Thin Layer Chromatography. The hemolymph related to the presence of protein is known to yield pink spots with Ninhydrin. The presence of ninhydrin single positive spots indicated that the possibility of peptides is present in the hemolymph (*R. communis* fed *S. litura* hemolymph fraction I, Rf: 2.196; and *B. oleracea* fed *S. litura* hemolymph fraction II, Rf: 2.201) (Fig 6).

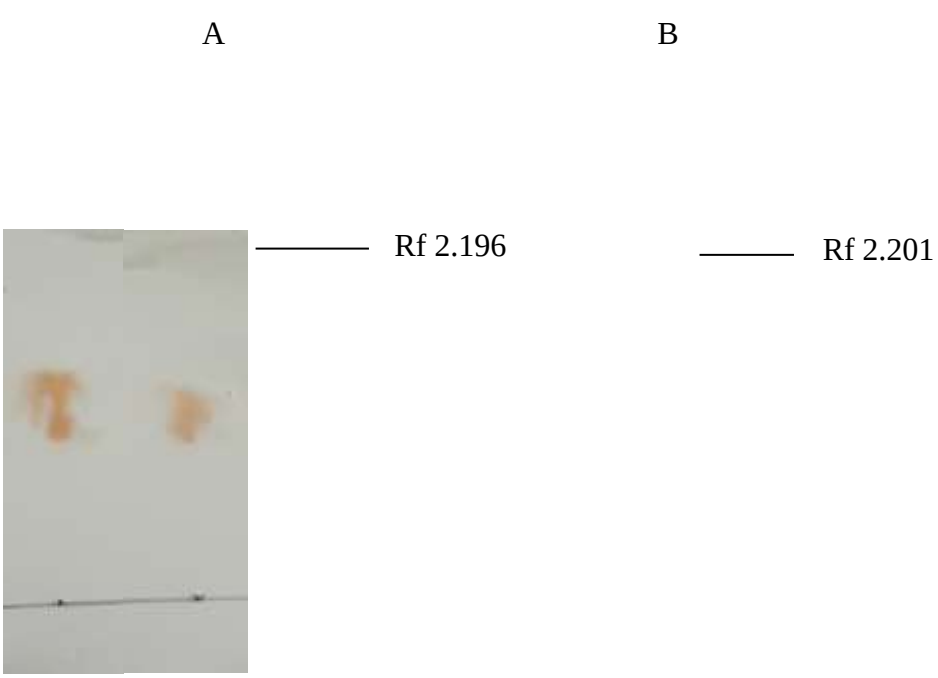


Figure 6: Analysis of purified peptide through TLC from *S. litura* larvae. A) *R. communis* fed fraction I, and B) *B. oleracea* fed fraction II.

SDS – PAGE

The scanning of the gel showed a separation of three protein bands (Fig 7). Highly expressed bands were observed in *R. communis* fed *S. litura* hemolymph fraction I whereas, *B. oleracea* fed *S. litura* hemolymph fraction II having similar bands but it does not show the high antimicrobial peptide expression. The molecular weight of purified peptide was near in 85-100 kDa.

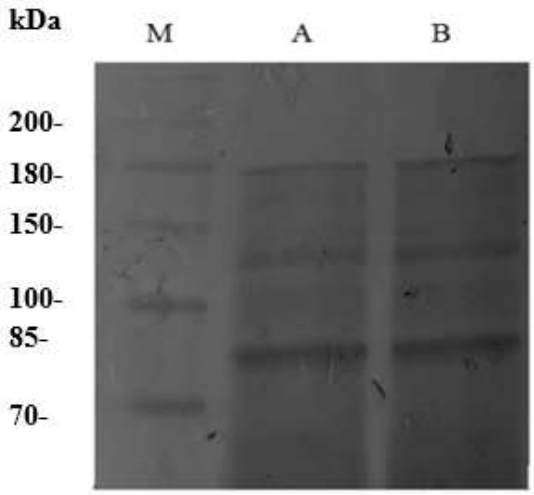
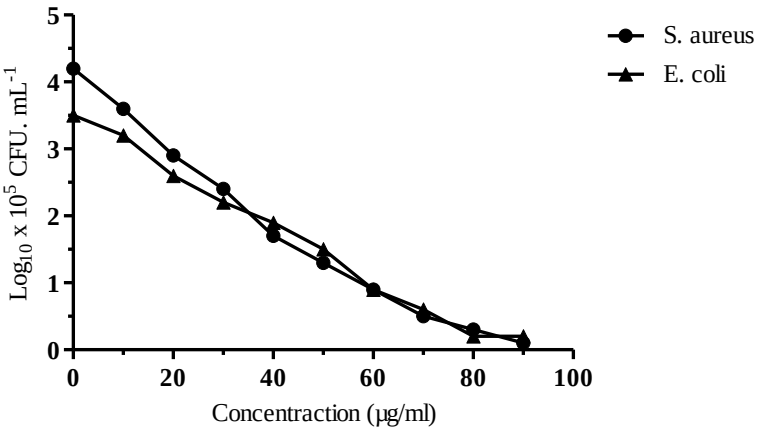


Figure 7: Analysis of purified peptide by SDS-PAGE. M indicates marker, A indicates *B. oleracea* fraction II, and B indicates *R. communis* fraction II.

Minimum inhibitory concentration

Highest antibacterial activity was observed on *S. aureus* and *E. coli*. The MIC value was determined against *S. aureus* and *E. coli* was found to be 90 µg/ml in *R. communis* fed *S. litura* hemolymph fraction I (Fig. 8A). The MIC value was found to be 70 µg/ml in *S. aureus* and 90 µg/ml of *E. coli* in *B. oleracea* fed *S. litura* hemolymph fraction II (Fig. 8B).

A



B

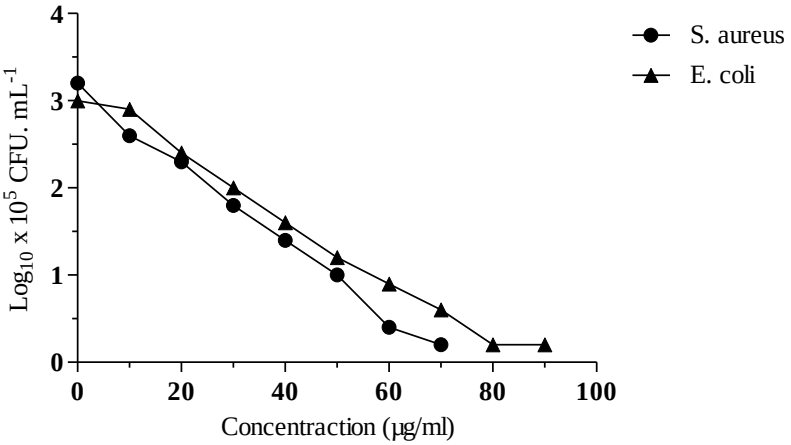
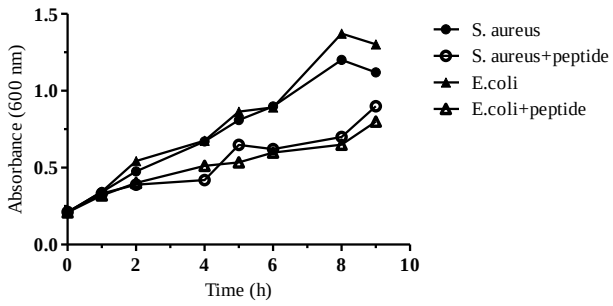


Figure 8: Effect of different concentration of purified peptide on the growth of Gram positive and Gram negative bacteria. *S. aureus* and *E. coli* to determine MIC value. A) *R. communis* fed fraction I, and B) *B. oleracea* fed fraction II.

The growth curve of *S. aureus* and *E. coli* was prepared in the presence and absence of peptide. Microbial growth was assessed with the peptide at 32 µg/ml. The best growth curve was observed in *R. communis* fed *S. litura* hemolymph fraction I (Fig.9).

A



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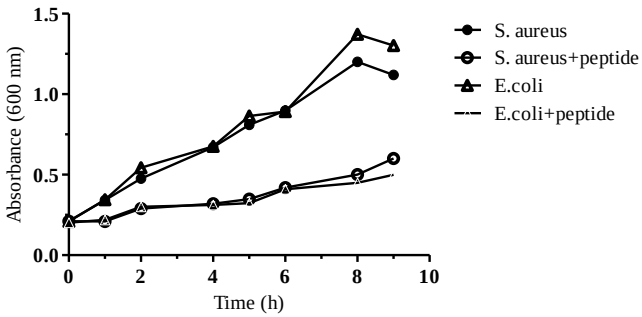

Figure 9: Growth curve of *S.aureus* and *E.coli* in the presence or absence of the sub inhibitory concentration of purified peptide. Closed circle and empty circle represent the growth of *S.aureus* in the absence and presence of peptide. Closed triangle and empty triangle represent the growth of *E.coli* in the absence and presence of peptide. A) *R. communis* fed fraction I, and B) *B. oleracea* fed fraction II.

Discussion

It is known that *B. thuringiensis* producing crystals protein and with having insecticidal properties. These toxins are produced strong insecticidal activity in many lepidopteran pests. Nowadays, insects produced many virulence factors that were identified against microbial exposure. According to Li et al. (2018) has been reported more AMP resistant factors were identified in *Plutella xylostella* larvae against *B. thuringiensis* strains. AMP and lysozyme are acted as chemical effectors to arrest microbial infections (Lemaitre and Hoffmann, 2007). In insect, AMP is expressed in many gene families like cecropin and drosomycin are well characterized in *Drosophila melanogaster* (Sackton et al. 2007) and cecropin, moricin, and gloverin identified in *Bombyx mori* (Cheng et al. 2006).

Brogden (2005) has been suggested that the mode of action of AMP and kill the bacteria varied by disrupting the cell membranes, interfering with metabolism, and targeting cytoplasmic components. Antimicrobial peptides are produced by various binding models includes barrel-stave, carpet or toroidal-pore. These models allow them to penetrate the cell to inhibition of cell wall synthesis, changes cytoplasm membrane, activation of autolysin, the central dogma of DNA, RNA, protein synthesis and also certain enzymes. The antimicrobial potential of hemolymph collected from the *Spodoptera litura* exerts strong activity against the tested microbes. It has been observed that in various invertebrate species by exposure of bacteria into the hemocoel elicit the synthesis of several antimicrobial peptides and proteins, which are recreated into the hemolymph and are active (Gillespie et al., 1997). As the insect hemolymph samples showed that antibacterial activity, suggest that a broad spectrum of antibacterial peptides was secreted in response to immunization (Hoq et al., 2003). In this study, purified both hemolymph samples by reverse- phase HPLC with a linear gradient. The purity of the final purified was judged by HPLC. The high peak was observed in both experimental groups and also done conformation of peptides present in the hemolymph. Growth-inhibition zone assay suggested that the purified peptides from *S. litura* larvae have antimicrobial activities against pathogenic microorganisms including Gram-positive bacteria

and gram-negative bacteria. The isolated peptide was showed the best inhibition growth in *S. aureus* and *E. coli*. *S. aureus* responsible for food poisoning through the production of enterotoxin (Whitt and Salyers, 2002) and *E. coli*, which causes gastroenteritis, urinary tract infections and meningitis (Todar, 2007). Therefore our findings, Gram-positive bacteria were shows more inhibition activity than the Gram-negative bacteria. We suggesting that the production of antimicrobial peptides are main defense mechanisms against the microbes which can control bacterial growth also.

Antioxidant activity deals with the measurement of the total antioxidant capacity present in the sample. Its play an important role in insect defense mechanism against toxins (Beutler, 2014; Slowinska et al., 2016). Here the total antioxidant was high in *R. communis* fraction I when compared to other treated fractions. Slowinska et al. 2016 have reported antioxidant capacity was increased in honeybee *Apis mellifera* hemolymph when the exposure of toxins.

Lysozyme is most effective against gram-positive bacteria since the peptidoglycan layer is relatively accessible to the enzyme. Lysozyme is effective against gram-negative bacteria only after the outer membrane has been compromised. Lysozyme is not thought to be involved in destroying gram-negative bacteria. The lepidopteran lysozyme genes are induced by bacterial challenge and lysozyme activity in insect hemolymph, maintained constitutively at a low level after bacterial infection increases considerably (Chung and Ourth, 2000; Lavine et al., 2005). Our result was investigated whether the level of lysozyme activity changed after treatment with *B. thuringiensis*. It is known that lysozyme plays an important role in the humoral defense of *Spodoptera litura*. Total Lysozyme activity was measured from *S. litura* larvae in different concentrations of purified peptide. Hemolymph incubated with *M. luteus*, the activity of lysozyme level was increased in both in the case of hemolymph was observed in *R. communis* fraction I and *B. oleracea* fraction II. Therefore the lysozyme present in the hemolymph will degrade the cell walls.

Novel AMP to develop the drug in low toxicity against human blood erythrocytes. This measurement generally by the ability of AMP to lyse human red blood cells and also other mammalian red blood cells were used for analysis sheep (Ryge et al., 2004), rat (Krishnakumar et al., 1999), pig (Dennison and Phoenix, 2014), and rabbit (Liu et al., 2012). These studies indicated that hemolytic activity strongly depends on mammalian erythrocytes. Here, the hemolytic activity done by goat erythrocytes, the percentage of hemolysis was very low in *R. communis* fraction I.

In this present study antimicrobial peptides purity checking by using Thin Layer Chromatography. Pink color spots were indicating the possibility of containing peptides. Reports the isolation of different peptides by using SDS-PAGE among which a peptide of molecular weight near 85-100 kDa was found. This peptide described in many lepidopteran insects like, *H. armigera* (Mackintosh et al., 1998), *T. ni* (Lundstrom et al., 2002), *G. mellonella* (Seitz et al., 2003), *Antheraea mylitta* (Gandhe et al., 2006), *M. sexta* (Abdel-latief and Hilker, 2008; Xu et al., 2012), *Diatraea saccharialis* (Silva et al., 2010), *Plutella xylostella* (Etebari et al., 2011), *S. exigua* (Hwang and Kim, 2011), and *B. mori* (Kawaoka et al., 2008) and first it was isolated in *Hyalophora gloveri* pupae (Axen et al., 1997). A similar phenomenon has been observed in silk worm, *Antheraea assamensis* (Nayak, et al., 2018). Therefore the identified possible peptide are thought to participate in immune responses against microbial infections.

Conclusion

Production of AMP is an important immune response against the infection of invading microorganisms because AMPs have broad-spectrum antimicrobial activities. Studies on identification and functioning mechanism of possible AMP have been extensively investigated in invertebrates. The isolated peptide form *R. communis* fed *S. litura* hemolymph fraction I and *B. oleracea* fed *S. litura* hemolymph fraction II has high antimicrobial activity, lysozyme activity, total antioxidant, and less lytic activity. *S. litura* hemolymph increased significantly and expression levels of peptides induced highly after the injection with bacteria. The presence of peptide suggests that they play a vital role in the microbial infection in *S. litura*. The simultaneous presence of peptides in immune hemolymph suggests that they comprise a part of a peptides involved in fighting against infection in *Spodoptera litura*.

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