6. SUMMARY

Study on antibacterial proteins in invertebrates in recent times have been of immense interest, as these molecules take part in important function of innate immunity. Such antibacterial molecules with bacteriolytic functions have been isolated from various organisms including insects. Lytic actions of some of these proteins have been described to the activity of enzymes, a change in normal state of the actions of membrane-associated enzymes or pore formation in the membrane of target organisms. The present study entitled “Detection, purification and characterization of a potent bacteriolytic factor from the larvae of blowfly, Chrysomya megacephala” was carried out with appropriate screening, identification and purification of a lytic molecule to understand its molecular and functional nature. The following are the salient findings of the present research investigation:

CHAPTER -1:

In the present study, for the first time to our knowledge of understanding, a lytic molecule was detected in the larval extract of blowfly, C. megacephala. The basic features of lytic molecule from the larval extract of blowfly was characterized in this chapter using assays such as erythrocye lytic (hemolytic) activity, cation dependency, EDTA sensitivity, pH stability, thermal tolerance, sensitivity to proteolytic enzymes, response to protease inhibitor and inhibition of lytic (hemolytic) activity by specific carbohydrates.

- As an initial step in the detection of lytic molecule, the crude larval extract of C. megacephala was subjected to hemolytic assay using various vertebrate RBC types viz., ox, buffalo, sheep, rat, human-A, B, O, cow, goat, mouse and hen. All tested RBC types were lysed by the 10 % crude larval extract. Among them, the highest hemolysis of 99.04 % was obtained with ox RBC followed by 72.2 % for cow RBC. Rat, buffalo and sheep
RBC types showed similar value of hemolysis as 64 %. Hen, goat and human-O RBC types were lysed by the larval extract with a comparable hemolysis value of 59 % without any discrimination among these three RBC types. Similarly, the mouse and human-B RBC types were lysed by the larval extract at a percent of 57.93 and 54.36 respectively. A low level (37.59 %) of hemolysis was observed with human-A RBC type.

- In cross adsorption test, adsorptions of larval extract of *C. megacephala* thrice subsequently with fixed ox RBC (erythrocyte type with highest percentage of hemolysis) was sufficient to completely remove the hemolytic activity from the larval extract. The residue of larval extract adsorbed with fixed ox RBC type was failed to lyse native (unfixed) ox RBC type as well as other native vertebrate RBC types. Subsequently, erythrocyte type with highest percentage of hemolysis i.e., ox RBC was chosen as suitable indicator cells for all subsequent experiments.

- The hemolytic activity of larval extract of *C. megacephala* against indicator RBC type was found to be relatively stable between pH 4 and 10, reduced at pH below or above this pH range and completely lost at pH 3 and pH 11. Thus, it showed a preference to neutral pH for lytic activity of the crude larval extract.

- The hemolytic activity of the larval extract was unaffected for the indicator RBC type up to 50°C. It was reduced considerably at higher temperatures and completely inactivated at 80°C. This showed moderate thermo tolerance of the crude larval extract for its lytic activity.

- The larval extract of *C. megacephala* showed significantly higher hemolytic activity in the presence of cations such as K⁺, Ca²⁺, Mn²⁺ and Mg²⁺. Among the cations tested, hemolytic activity was higher (99.04 %) in
the presence of Ca\textsuperscript{2+}, while the hemolytic activity of larval extract was reversibly inhibited by EDTA. This suggested that the hemolytic activity of larval extract of \textit{C. megacephala} was dependent on mono and divalent cations with its high dependence on Ca\textsuperscript{2+} and also highly sensitive to EDTA.

- Presence of hemolytic activity in the precipitation obtained with 75 \% ammonium sulphate or supernatant obtained after precipitating the protein with 10 \% TCA against indicator RBC specified that the molecule responsible for larval extract hemolytic activity appeared to be proteinaceous in nature.

- The proteinaceous nature of hemolytic activity of larval extract was examined by exposing the extract to proteolytic enzymes. The results of these experiments clearly indicated that the molecule responsible for hemolytic activity against indicator RBC type was shown to be highly sensitive to pronase and moderately resistant to trypsin and \( \alpha \)-chymotrypsin.

- The hemolytic activity of larval extract was treated with a well known protease inhibitor, phenylmethylsulphonyl fluoride (1mM PMSF) to relate its role as protease (enzyme). The results showed no inhibition of the hemolytic activity in the larval extracts signifying that the molecule responsible for larval extract hemolytic activity was proteinaceous and not enzymic in nature.

- Effect of cation chelator on the hemolytic activity of the larval extract was tested using different concentrations of EDTA. The results of these experiments revealed that the hemolytic activity of larval extract was sensitive to a concentration of 10 mM EDTA.
Hemolytic inhibition assay was performed with 31 sugars, 5 polysaccharides and a glycoprotein. Among the various carbohydrates and glycoprotein tested, the inhibition of hemolytic activity of larval extract of *C. megacephala* was highly specific to *N*-acetyl-neuraminic acid (6.25 mM NANA) with a hemolytic inhibition of 86.55 % and fetuin (0.25 %) with a hemolytic inhibition of 59.85 %. Moderate hemolytic inhibition of larval extract was observed with lactose (39.32%), cellobiose (24.17 %), chitin (37.98 %) and chitosan (37.98 %). None of the other carbohydrates showed any inhibitory effect on the hemolytic activity of larval extract of *C. megacephala* against indicator RBC type.

Over all, this preliminary study revealed the presence of a lytic molecule in the larval extract of *C. megacephala* with maximum hemolytic activity for ox RBC. This hemolytic molecule was dependent upon cations for its activity and sensitive to EDTA. The hemolytic activity of larval extract was heat-labile and stable at neutral buffer. It was highly sensitive to pronase and moderately resistant to trypsin and *α*-chymotrypsin. The molecule responsible for larval extract hemolytic activity appeared to be proteinaceous in nature. The lytic activity of larval extract was highly specific to *N*-acetyl-neuraminic acid (NANA) and fetuin.

**CHAPTER -2:**

In this chapter, experiments were carried out on the purification and molecular characterization of hemolytic molecule in the larval extract of *C. megacephala* through batch assays with various column matrices, purification by batch adsorption method and analysis of purified protein by electrophoresis. In addition, the purified protein was subjected to analyses such as UV-visible absorption spectrum, Fast Phase Liquid Chromatography (FPLC), two dimensional (2-D) gel electrophoresis, peptide mass fingerprinting, HPLC and circular dichroism (CD) spectrum. Besides, the essential features of purified lectin
were characterized with various experiments as performed for preliminary characterization.

- Preliminary studies on the inhibition of hemolytic activity from larval extract of *C. megacephala* revealed significantly higher inhibitory reaction by *N*-acetyl-neuraminic acid and moderate inhibitory reaction by lactose. The binding ability of hemolytic molecule in crude larval extract was therefore verified using epoxy activated lactose-Sepharose CL-6B and glutaraldehyde fixed indicator (ox) RBC as affinity matrices through batch assay. Among these two matrices tested for the adsorption of hemolytic molecule from crude larval extract, fixed ox RBC (*N*-acetyl-neuraminic acid is a component of glycoprotein found predominantly as terminal sugar on the erythrocyte membrane of vertebrates) was found to be efficiently adsorbed all the lytic activity.

- Glutaraldehyde fixed ox RBC was therefore selected for further purification of hemolytic molecule from the larval extracts of *C. megacephala* by batch adsorption method.

- The spectrophotometric analysis of batch adsorbed and eluted fractions showed a symmetrical peak as well as coincident peak of hemolytic activity against indicator RBC. This batch adsorption procedure adopted for the purification of lytic molecule resulted in about 81 % recovery of hemolytic activity.

- The purified lytic protein on native gel electrophoresis revealed a single protein band in 8 % acrylamide gel stained with coomassie brilliant blue as well as silver nitrate. On the other hand, denatured gel electrophoretic (SDS-PAGE) analysis of purified protein showed four polypeptide subunits on a 10 % acrylamide gel. The molecular weight of major polypeptide
subunit was approximately 75 ± 2 kDa and other three polypeptides were approximately 69 ± 8 kDa, 61 ± 7 kDa and 55 ± 9 kDa. The molecular weight of purified hemolytic protein thus calculated using the molecular weights of four polypeptide subunits was approximately 260 ± 26 kDa.

- The profile of absorption spectrum of purified protein revealed a single peak with an absorption maximum at 280 nm without any contamination.

- The purified hemolytic molecule subjected to FPLC in gel filtration on a column of Superose 6 10/300 GL disclosed a single symmetrical peak with a retention time of 28.39 min. The calculated molecular weight of this purified hemolytic molecule was approximately 290 kDa as estimated from the standard curve obtained for known molecular weight markers versus retention time. This molecular weight was almost matched with a size of 260 ± 26 kDa as calculated from the polypeptide subunits obtained through SDS-PAGE.

- The purified hemolytic molecule was subjected to 2-D gel electrophoresis. The first dimension was carried out with the purified protein on IEF strips with linear pH gradient of 3 to 10. The second dimension was done on an IEF gel using 10 % SDS-PAGE. Purified protein in the second dimension was resolved into four polypeptides with a major subunit of approximately 75 kDa at the isoelectric point of 8 and three minor / less intensely stained subunits at neutral pI of 7 (69 kDa) and acidic pI of 5.2 (61 kDa) and 4.3 (55kDa).

- The MALDI-TOF-MS/MS and mascot analyses of trypsin digested 75 kDa polypeptide subunit of the purified molecule showed maximum matches with amino acid sequences of molecules with hemolytic and antimicrobial nature isolated earlier from the venom of few hymenopteran insects.
The predicted short amino acid sequences of peptides obtained for 75 kDa polypeptide subunit of the purified molecule showed significant homology in the range of 60-100 % on the conserved and semi-conserved substitution regions of bacteriolytic peptide isolated from dipteran insects. It was also comparable with amino acid sequences of antimicrobial molecules (diptericin) from the larvae of Calliphoridae (blow flies) and Sarcophagidae (flesh flies) with a significant homology in the range of 41-100 %. Similarly, the peptide sequence of the fragment with m/z ratio - 2191.0110 revealed significant homology (21-100 %) on the conserved and semi-conserved substitution regions of C-type lectin (from venom of gabon viper) and hemolytic lectin (from marine invertebrates and dipteran insects). Furthermore, the predicted amino acid sequences aligned with amino acid sequences of peptidoglycan recognition protein of other dipteran insects revealed its significant homology in the range of 23-100 %. This analysis confirmed that the purified molecule from the larval extract of C. megacephala could be hemolytic, antibacterial / bacteriolytic and carbohydrate binding in nature.

- The HPLC chromatogram obtained for the purified protein showed presence of 13 different amino acids. Among the residues of various amino acids, leucine was detected with a maximum quantity of 71.6 % over other amino acids.

- Circular dichroism (CD) spectrum of the purified protein of C. megacephala was obtained at 190 to 250 nm (far UV) and 250 to 350 nm (near UV) range. The spectrum with a positive peak at 190 nm predicted the presence of helical nature (20.4 %). An intense negative peak from 208-220 nm showing an indication for dominant occurrence of β-pleated sheets (49.9 %) in the purified protein. A very little variance observed in near UV spectra was suggesting very low aromatic amino acid
content in purified lytic protein. A ratio of 49.9 % of β-sheets, 20.4 % of α-helices and 29.8 % of random coil or connecting lube were obtained with the secondary structure prediction of purified hemolytic protein.

- The hemolytic activity of the purified protein from the larval extract of *C. megacephala* was tested against eleven vertebrate RBC types. As observed with crude extract, almost the same pattern of hemolytic activity was observed in all those RBC types. The highest hemolysis of 94.56 % was observed with indicator (ox) RBC type. The lytic activity was increased with increasing concentrations of purified protein. A maximum hemolytic activity was observed as 89 % for 0.523 mg ml⁻¹ of purified protein. It was observed as minimum as 12.40 % for 0.008 mg ml⁻¹ of purified protein. The hemolytic activity of purified protein (0.523 mg ml⁻¹) was observed against indicator RBC over a period of 80 minutes. An increase upto 80 % in hemolysis was observed after an incubation time of 40 minutes and 82.56 % of hemolysis after 80 minutes.

- The hemolytic activity of purified protein was constant between pH 4 and 10, and preference for its lytic activity was noticed towards neutral pH. The hemolytic activity of purified protein was unaffected up to 40°C for the indicator RBC type. But it was reduced considerably at higher temperatures and completely inactivated at 80°C. It showed moderate thermo tolerance. The purified protein was dependent on cations (Preferably calcium) for its complete hemolytic activity and also sensitive to EDTA.

- The hemolytic activity of purified protein from larval extract *C. megacephala* was found to be highly sensitive to pronase and moderately resistant to trypsin and α-chymotrypsin. Purified protein treated with heat-inactivated proteolytic enzymes failed to affect the hemolytic
activity of purified molecule against indicator RBC type. Consequently, to relate the hemolytic activity of purified molecule to protease, it was treated with a well known protease inhibitor, PMSF (1mM). The results revealed no inhibition of hemolytic activity by PMSF and showing 80.45% of hemolytic activity against indicator RBC. These results thus indicating proteinaceous nature of purified lytic molecule from the crude larval extract of *C. megacephala*.

- The inhibition of hemolytic activity of purified protein was highly specific to *N*-acetyl-neuraminic acid (NANA) with a hemolytic inhibition of 89.63%. Moderate hemolytic inhibition of purified protein was observed with fetuin and lactose, a low hemolytic activity was recorded with chitin and chitosan and almost no hemolytic activity was known for cellobiose. These results were almost similar like that of the results obtained for crude larval extract.

- Scanning Electron Microscopic analysis of untreated (normal) indicator (ox) RBC revealed a smooth surface of cells with maintenance of their biconcave discoid shape. These erythrocytes treated with purified hemolytic protein revealed shrunken and distorted nature of cells. In addition, a well marked visible holes, disruption of RBC membrane and oozed out cytoplasmic content were found in these erythrocytes.

On the whole, purification of lytic protein from the larval extract of *C. megacephala* using glutaraldehyde fixed ox RBC by batch adsorption method showed a single protein without any contamination in native-PAGE. It was confirmed by FPLC analysis with a single peak of a fraction with molecular weight of 290 kDa. SDS-PAGE analysis of purified protein showed four clear polypeptide subunits with approximate molecular weight of 75 ± 2 kDa, 69 ± 8 kDa, 61 ± 7 kDa and 55 ± 9 kDa. The molecular weight of purified fraction
thus calculated using the molecular weights of four polypeptide subunits was approximately 260 ± 26 kDa. The amino acid sequences of short peptides obtained out of mass spectrophotometric analysis of purified lytic protein revealed its homology with amino acid sequences of molecules with hemolytic, antibacterial / bacteriolytic and sugar binding nature. The inhibition of hemolytic activity of purified protein from the larval extract of *C. megacephala* was highly specific to *N*-acetyl-neuraminic acid with a hemolytic inhibition of 89.63 %. The purified molecule treated with enzymes and PMSF indicated proteinaceous nature of the lytic molecule. HPLC analysis of the purified lytic molecule revealed leucine as a predominant amino acid residue with a maximum quantity of 71.6 % among various other amino acids detected. The secondary structure prediction of purified hemolytic protein using circular dichroism showed the molecule with 49.9 % of β-sheets, 20.4 % of α-helices and 29.8 % of random coil or connecting lube. The purified lytic molecule showed highest hemolysis of 94.56 % with indicator (ox) RBC. The hemolytic activity was increased with increasing concentrations of purified protein. A hemolysis of 82.56 % was observed after 80 minutes of incubation of purified protein at a concentration of 0.523 mg ml⁻¹ with the indicator RBC. The erythrocytes treated with purified lytic protein revealed shrunken and distorted nature of cells with a well marked visible holes, disruption of RBC membrane and oozed out cytoplasmic content. Almost similar results were obtained on the basic characteristic features of the purified lytic protein as that of studies carried out for preliminary characterization using crude larval extract.

**CHAPTER -3:**

This chapter deals with the functional characterization of purified lytic protein from larval extract of blow fly *Chrysomya megacephala*. Analysis of antibacterial activity, especially bacteriolytic property of both crude larval extract and purified lytic protein was carried out with two laboratory bacterial cultures and ten soil bacteria obtained from native larval habitat of *C. megacephala*. 
Ten different bacterial colonies were isolated from the soil of larval habitat of *C. megacephala* based on the growth and morphology of bacterial colonies on agar plate.

The antibacterial property of crude larval extract as well as purified protein was verified using “zone of inhibition (ZI) assay” utilizing two laboratory bacterial colonies viz., *Micrococcus luteus* MTCC 106 (Gram-positive), *Escherichia coli* MTCC 443 (Gram-negative) and ten different bacterial colonies isolated from the larval habitat of *C. megacephala*.

Out of twelve bacteria tested for antibacterial activity, the growth of soil bacterial isolates IV (Gram-positive), VIII (Gram-negative) and laboratory bacterial colonies of *Micrococcus luteus* MTCC 106 (Gram-positive) and *Escherichia coli* MTCC 443 (Gram-negative) were only inhibited by the crude extract as well as purified protein.

The crude larval extract (1.27 mg of protein) inhibited the growth of bacterial isolates IV, VIII, *M. luteus* and *E. coli* with the zone of inhibition of 10 ± 0.5 mm, 8 ± 0.5 mm, 7 ± 0.5 and 8 ± 1.0, respectively. Likewise, the purified protein (26.15 µg) was also inhibited the growth of soil bacterial isolates IV, VIII, *M. luteus* and *E. coli* with a zone of inhibition of 7 ± 0.5 mm, 7 ± 0.5 mm, 6 ± 1.0 mm and 7 ± 0.5 mm, respectively.

The soil bacterial isolates IV and VIII subjected to morphological and biochemical characterizations including molecular analysis revealed its identity as *Paenalcaligenes hermetiae* (GenBank Acc No: KX463630) and *Enterococcus casseliflavus* (GenBank Acc No: KX417299).

Tests were conducted to determine the bacteriolytic activity of purified protein. The bacteriolytic activity was increased with increasing
concentrations of purified protein from the larval extract of *C. megacephala*. For example, the purified protein at a concentration of 0.523 mg ml$^{-1}$ was tested for its bacteriolytic activity against four different sensitive bacterial species. At this concentration, a maximum bacteriolytic activity of 74.70% was observed in the case of *E. coli*. A bacteriolyasis of 63.74% was recorded for *Paenalcaligenes hermetiae* (soil bacterial isolate IV) and 37.5% was noticed for *Enterococcus casseliflavus* (soil bacterial isolate VIII). It was observed as a minimum bacteriolysis of 24.41% in the laboratory culture of *M. luteus*.

- The membrane integrity of bacterial cells was determined in terms of measuring the amount of intracellular materials/components released from the cells subjected to the treatment of purified protein at different time intervals. The bacterial colonies such as *P. hermetiae* (bacterial isolate IV), *E. casseliflavus* (bacterial isolate VIII), *M. luteus* and *E. coli* treated with the purified protein (52.3 µg / 100 µl) showed an increase in the absorbance at 260 nm after 20 min of incubation. The increased absorbance due to release of intracellular material (after removing cell debris) clearly indicating the lysis of tested bacterial cells by the purified protein.

- The bacteriolytic activity of purified protein was analyzed using SEM with Gram-negative bacterial strain of *Paenalcaligenes hermetiae* (sensitive bacterial isolate obtained from larval habitat). The normal bacterial cells revealed a smooth surface with maintenance of their rod shape. Shrunken and distorted (structurally altered) cells were observed in bacteria treated with purified protein from the crude larval extracts of *C. megacephala*. In addition, visible holes and disruption of membrane were found in bacterial cells treated with purified protein.
• Evaluation of bacteriolytic property of crude (1.27 mg of protein) as well as purified protein (26.15 μg) from the larval extract of *C. megacephala* was performed with chromogenic bacteriolytic plate assay using *Escherichia coli* strain BL21. The results revealed formation of sharp blue circle staining / halo along the edge of the inhibition zone produced by crude larval extract (8 ± 1.0 mm blue halo) and purified protein (7 ± 0.0.5 mm blue halo) confirmed the bacteriolytic nature of crude and purified protein from the larval extract of *C. megacephala*.

Significant findings on the study of functional characterization revealed prospective bacteriolytic activities of the purified lytic protein from the larval extract of blow fly *Chrysomya megacephala* for two Gram-positive bacteria - *Enterococcus casseliflavus, Micrococcus luteus* and two Gram-negative bacteria - *Paenalcaligenes hermetiae* and *Escherichia coli*. Presence of α-helical propensity and strong hydrophobic interaction due to richness in leucine residues in the purified molecule could be a possible mechanism of action for its lytic activity on various erythrocytes and bacterial cells as reported in various studies.