1. INTRODUCTION

1.1 Insect immunity

Insects have been extraordinarily successful organisms among other living beings on earth during the course of evolution. Current estimates revealed the fact that insects account for more than 70% of entire existing animal species on earth with their inhabitance on all ecological niches. Like other living organisms they are also challenged by an extremely large variety of potentially harmful pathogenic microorganisms. The first-line defense barriers combating such harmful pathogens in insects includes: outer exoskeleton, peritrophic matrix of midgut epithelium and chitinous lining of trachea. But, these physical barriers cannot prevent penetration and subsequent infection of pathogenic microorganisms. Once microbes enter the haemocoel of insects, they are exposed to responses like humoral and cellular defense reactions (Leclerc and Reichhart, 2004; Waterfield et al., 2004; Abraham and Jacobs-Lorena, 2004). It is very well known that insects have evolved effective cellular and humoral immune mechanisms (Ratcliffe and Rowley, 1979) and are capable of acquiring a protected (immune) state after surviving bacterial infections by secreting a series of antimicrobial molecules into the hemolymph (Gotz and Boman, 1985; Ratcliffe et al., 1985; Brehelin, 1986; Dunn, 1986). Humoral defensive mechanism includes production of antimicrobial peptides, induction of molecules like lectin and activation of the pro-PO system, whereas, cellular defenses involve hemocyte
mediated immune reactions that comprise phagocytosis, nodulation and encapsulation (Hoffmann, 2003; Iwasaki and Medzhitov, 2004). The innate defense system in insects is essential for the survival and maintenance of all multicellular organisms (Hoffmann et al., 1999; Salzet, 2001).

There are two major factors of instigating an event of proteolytic cascade involved in innate defense in insects. First initiation factor is the formation of localized blood clotting followed by melanization at the site of injury and around invading microorganisms. The second factor is development of phagocytic mechanism against microorganisms through specialized cells, or through formation of capsules around larger sized parasites by certain blood cells. Concurrently, the synthesis of a battery of effective, generally small-sized antimicrobial peptides is induced predominantly in the fat body of insects. These peptides are released into the hemolymph and involve in killing the invading microorganisms. Recognition of infectious non-self molecules is mediated by means of proteins in insects both in circulation and on cell membranes that bind preferentially to microbial cell wall components. They bind on lipids and carbohydrates which are synthesized by the microorganisms and are exposed on their surface as cell wall components, such as lipopolysaccharides (LPS) of Gram-negative bacteria, lipoteichoic acids (LTA) and peptidoglycans of Gram-positive bacteria and β-1, 3-glucans of fungi (Nappi et al., 2000). These recognition proteins are referred as pattern recognition receptors (PRRs) in insects (Hoffmann, 1995). The recognition of a pathogen is the starting point in the establishment of
immune response that is either through humoral or cellular mechanisms. This is achieved by these pattern recognition receptors (PRRs) or pattern recognition proteins (PRPs) that identify and bind to conserved domains (patterns) found on the membrane surface of a pathogen, which are described as pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1997).

The most characterized PRPs are the C-type lectins, peptidoglycan recognizing proteins, β-1, 3-glucan proteins, hemolins, integrins etc., (Bettencourt et al., 1997; Michel et al., 2001; Bettencourt et al., 2004). The binding of invading PAMPs on PRPs induces synthesis of antimicrobial proteins or initiates the proteolytic activation of phenoloxidase flow or turn on cellular immune response, guiding to phagocytosis, nodule formation and encapsulation of the invaders (Yu et al., 2002; Marmaras and Lampropoulou, 2009). The recognition of invading pathogen either as bacteria or fungi or even viruses is followed by the immediate de novo synthesis of antimicrobial peptides (AMPs) and their secretion into the hemolymph (Zasloff, 2002; Bulet et al., 2004). These peptides are mainly synthesized by the fat body and in a lesser degree by the hemocytes, integument, gut, salivary glands and reproductive structures (Nappi and Ottaviani, 2000). Over 150 antimicrobial peptides (AMPs) have been isolated and characterized so far in insects. These molecules are small cationic peptides, which bind to anionic bacterial or fungal membranes leading to disruption and cell death (Zasloff, 2002; Yount and Yeaman, 2004). Presence of several antimicrobial molecules acting in synergy is responsible in insects for a more powerful defense towards harmful invaders like bacteria, fungi and protozoa (Bulet et al., 1999).
1.2 Major insect pathogens

Bacteria constitute a large domain of microorganisms, due to their fast growth rate, frequencies of genetic mutations and selections, their ability to rapidly exchange genes and bacterial resistance to antibiotics seems to take place more quickly in the evolution of their development (Fredrickson et al., 2004; Bulet and Stöcklin, 2005). The global expansion of bacteria with antibiotic resistance is a major threat. Despite great progress in better understanding of the resistance mechanisms, the solution to this problem remains elusive (Laxminarayan et al., 2013). Several new bacterial diseases are highly prevalent in recent years due to many multi-drug resistant strains of bacteria that are often not sensitive to classical antibiotic treatments. In this context, there is an essential need for exploration of new generation of antibiotics and alternate treatments. For decades, one major area of interest for discovery and study of new antibiotics was the investigation of AMPs derived from insect immune defence reactions (Bulet and Stöcklin, 2005). There is a considerable variation in the number of AMPs in insects among various species (Vilcinskas et al., 2013). There is also a known chance of working together of the functionally distinct insect AMPs when expressed concurrently during an innate immune response (Brogden, 2005). Insect AMPs are certainly co-expressed (Riddell et al., 2011; Barribeau et al., 2014; Poppel et al., 2015) and naturally co-occurring AMPs display potentiating effects on bacterial pathogens (Engstrom et al., 1984; Hara and Yamakawa, 1995).
1.3 Insect antimicrobial molecules

In general, the insect antimicrobial molecules have a broad range of activities. They include, change in electrochemical gradients through transmembrane which is essential for microbial homeostasis, inhibiting protein synthesis, inducing membrane permeabilization and rupture, or promoting synthesis of reactive oxygen species that cause cell death without any cytotoxicity. They can be measured as excellent candidates to overcome the alarming problem of acquired bacterial drug-resistance and emergence of opportunistic pathogenic organisms, particularly in immune-suppressed hosts (Bulet et al., 1999; Thevissen et al., 2004; Rahnamaeian, 2011). Among the humoral substances, antibacterial peptides or proteins have been most extensively studied (Yeaton, 1981; Vasta et al., 1984; Olafsen, 1988; Smith and Chisholm, 1992; 2001) due to the following reasons: (1) they occur in the hemolymph or serum of almost every insect species studied and (2) they interact directly with foreign materials, particularly with the potential microbial pathogens and thereby appear to serve as humoral recognition function in second line of defense. Such proteins have shown to possess antimicrobial activity include lysozymes, metal-binding proteins such as transferrins, agglutinins, acute phase proteins, hemocyanins and antimicrobial peptides (Smith and Chisholm, 2001). These hemolymph proteins have been shown to cause either bactericidal, bacteriolytic or bacteriostatic activities or bacterial agglutinating activities (Adams, 1991; Destoumieux et al., 1999). Among them, the bacteriolytic protein is a heat stable and relatively low molecular weight
lytic protein, which is one of the inducible serum substances capable of lysing certain Gram-negative bacteria perhaps that combines with bacterial cell in the presence of complement, causing lysis or dissolution of the cells (Hultmark et al., 1980; Ginsburg, 2002). They probably function by deregulating phospholipids in Gram-negative bacteria and lipoteichoic acid (LTA) in Gram-positive bacteria (Ginsburg, 2002). Bacteriolytic activity in the hemolymph of grasshopper after injection of a *Bacillus poncei* was reported as early as 1918 by Glaser. There are three inducible bacteriolytic proteins that have been identified and designated as P7, P9A and P9B from the hemolymph of immunized pupae of the giant silk moth, *Hyalophora cecropia*. Based on the amino acid composition of the purified proteins, P7 showed a great similarity to that of the lysozyme from the wax moth *Galleria mellonella*. The other proteins, P9A and P9B are extremely powerful against *Escherichia coli* and a number of other Gram-negative bacteria. They are heat stable and their activities were retained after 30 minutes of incubation at 100°C. Among these two forms of proteins, P9 proteins clearly differ from the lysozyme class of enzymes and represent a new type of bacteriolytic protein (Hultmark et al., 1982).

### 1.4 Lytic / hemolytic molecules in insects

Agglutinins, lysins, phenoloxidase, lymphokine-like substances, lipopolysaccharide (LPS) binding proteins, clotting factors and some other proteins involve in antibacterial activities represent the most important humoral immune factors in invertebrates (Canicatti, 1990; Yu *et al*., 1999; Yu and Kanost,
2000; Yu et al., 2006; Xylander, 2009; Sasaki et al., 2010). Among these molecules, particular attention was always given to lysins, the biologically active substances characterized by their lytic properties against foreign targets. Hemolytic (HL) proteins are active against vertebrate erythrocytes, also suggesting that they perform similar lytic mechanisms against unwanted cells or non-self materials such as self-transformed cells, protozoa, parasites, bacteria etc., (Canicatti, 1990).

HL proteins have been detected from a variety of insect species. They are found in the venom (such as melittin, melittin-like hemolysin and barbatolysin) of social hymenopteran insects (Habermann and Jentsch, 1967; Schmidt and Blum, 1978; Bernheimer et al., 1980; Raghuraman and Chattopadhyay, 2007). Information on the presence of HL agents has been reported in some blood-feeding insects (Spates and Deloach, 1980; Kapatsa et al., 1989; Kirch et al., 1991). The physiological role of these factors is essential for these blood feeding insects for full utilization of the blood meal. In stable fly, Stomoxys calcitrans essential dietary components are associated with the lipid fraction on the membrane of erythrocytes (Kapatsa et al., 1989). Hence, hemolysis of erythrocytes is a requirement to meet nutritional condition of this hematophagous fly (Kirch et al., 1991). A HL activity (Gallysin-1) was identified in the larval hemolymph of the greater wax moth, Galleria mellonella (Phipps et al., 1989). In addition to its HL activity, Gallysin-1 damages the cell membranes of Gram-negative bacteria (Phipps et al., 1994). Gallysin-1 also exerts cytotoxic
activity against the human leukemia cell line, K562 (Beresford et al., 1997). Recently, Sasaki et al. (2010) reported the presence of HL protein in the hemolymph of the mosquito, Armigeres subalbatus.

1.5 Purification of hemolytic / cytolytic protein

A major problem in the functional characterization of the immunoglobulin-independent defense systems that lyse foreign cells, such as microbial pathogens is the purification of these cytolytic molecules (Canicatti, 1990; Wright and Unkeless, 1993). In invertebrates, recognition and lysis must be independent of immunoglobulin-based antibodies because antibodies are restricted to the vertebrates (Marchalonis and Schluter, 1990). There are only a few reports exist on the isolation and characterization of hemolytic factors in invertebrates. This is probably due to difficulties in maintaining the biological activity of the isolated molecules. In sea cucumber, Holothuria polii a chromatographic method was first employed to isolate hemolytic molecules. On a Bio-Gel A5m column, the coelomic fluid was resolved into three major components, the third of which showed lytic activity. This fraction when analyzed in electrophoresis under reducing conditions showed two polypeptide subunits with molecular weights of about 90 kDa and 68 kDa (Canicatti and Parrinello, 1983). Satisfactory results in the isolation of hemolytic molecule could be obtained when coelomocyte lysate is used instead of coelomic fluid. With an overlay technique two lytic bands which have different electrophoretic mobilities could be located by their biological activity. The components isolated and recognized were hemolysin-1 (He1) and
hemolysin-2 (He2). He1 is the calcium-dependent, heat-labile component whereas He2 is the calcium-independent and heat-stable molecule. The two hemolysins are probably two isoforms sharing a serological identity, as demonstrated by immunodiffusion analyses and have same molecular weight (80 kDa under non-reducing conditions; a doublet of 76 kDa and 80 kDa under reducing conditions) (Canicatti and Ciulla, 1988). Most of the studies were performed using techniques like gel filtration and ion exchange chromatography for the purification of these cytolytic molecules. Only few reports are documented using affinity (carbohydrate binding) based chromatography for the isolation of these cytolytic molecules (Armstrong et al., 1996; Sasaki et al., 2010).

1.6 Source of antimicrobial proteins or peptides in insects

The larval fat body (equivalent of the mammalian liver) is the largest organ of hemocoel and a major site of the intermediary metabolism in insects. It consists of thin layers or strings, generally one or two cells thick, or small nodules suspended in the hemocoel and distributed throughout insect body (Bullet et al., 1999; Roma et al., 2010). Majority of proteins in the hemolymph are synthesized in this tissue, which also serves as lipid, carbohydrate and protein storage, and is a major site for the production and secretion of antimicrobial proteins (Hoffmann, 2003). In the characterization of immune genes, upon induction of microbial infection, fat bodies encode several antimicrobial peptides. These molecules are then released into the hemolymph of insects (Hoffmann, 1995; Engström, 1998). In Drosophila, seven antibacterial (cytolytic) protein / peptides have been
characterized, namely, cecropin, attacin, defensin, drosocin, diptericin, metchnikowin including an antifungal peptide identified as drosomycin (Lemaitre and Hoffmann, 2007). In addition, Lepidopteran fat bodies synthesize and release numerous other proteins, such as pattern recognition proteins, hemolins and two immulectins, serine proteinases (for eg., prophenoloxidase activating proteinase) and a serine proteinase inhibitor from the serpin family (Zhu et al., 2003). It is well examined the nature of cells producing hemolytic molecules in the annelid Eisenia foetida. Results of studies in this worm (Valembois et al., 1982; Valembois et al., 1985) revealed that hemolytic molecules are synthesized and released from young chloragocytes and eleocytes. These hemolytic molecules are released in a short-term hemocyte culture medium in the mollusc, Mytilus edulis showed that they are actively secreted by the blood cells of this mollusc (Leippe and Renwrantz, 1988).

1.7 Surface binding and mechanism of action of hemolytic molecules

Hemolytic molecules are not reported to function as enzymes (Roch et al., 1981; Canicatti et al., 1987). It seems that hemolysis is mediated by the rapid binding of hemolytic molecules onto the target cells through sugars (Roch et al., 1981) or lipids (Canicatti, 1988). As demonstrated by Roch et al. (1981), the hemolytic system of E. foetida andrei is inhibited by various acetylated or methylated carbohydrates. Moreover, lipids also act as inhibitors of the hemolytic reaction (Roch et al., 1989). Carbohydrates are also found to be
inhibitors of hemolytic molecules in the *Strongylocentrotus droebachiensis* (sea urchin). In this species amino sugars are reported to be potent inhibitors, whereas acetyl-substituted amino sugars are less efficient (Bertheussen, 1983). In *H. polii* sugars did not inhibit the hemolytic system but lipids displayed inhibitory activity. In fact, sphingomyelin, one of the principal membrane lipids, inhibited hemolysis, which suggests a specific involvement of this lipid in the lysis of the target cell (Canicatti *et al.*, 1987; Canicatti, 1988). Sphingomyelin is also an inhibitor of the *Marthasterias glacialis* hemolytic molecule (Canicatti *et al.*, 1988). There is also a report of “limulin”, a lectin observed in one of the major cytolytic proteins in the plasma from *Limulus*. Presumably, the recognition of foreign cells for succeeding lytic destruction in this system depends on the presentation of sugars recognized by limulin on the surface of foreign cells. In this cytolytic lectin, hemolysis is dependent on the sialic acid-binding abilities of limulin, as the lytic process is inhibited by sialylated glycoconjugates and by desialylation of the target erythrocytes (Robey and Liu, 1981; Armstrong *et al.*, 1996).

1.8 **Role of hemolytic molecules in immune reactions**

Hemolytic molecules from different invertebrate species so far examined are active against vertebrate erythrocytes. The same lytic mechanisms are probably used against self-transformed cells, protozoa, parasites, bacteria etc. Moreover, hemolytic molecules could act as opsonic factors, alone or through proteolytic fragments produced by their activation. A clear indication of an opsonic activity of
the lytic proteins was demonstrated in the sea urchin *S. droebachiensis* (Bertheussen, 1983).

In *H. polii*, engagement of hemolytic molecule in the immune surveillance mechanisms was first indicated by injection experiments. When formalin fixed sheep erythrocytes were injected into the coelomic cavity, a decrease in the degree of hemolysis was registered in the injected samples compared to non-injected controls. This value increased steadily, reaching the control value on fourth day (Canicatti and Parrinello, 1985). The initial decrease is probably due to the involvement of hemolytic molecule in the clearance of foreign materials. It was supposed that these molecules could mediate as opsonins, the formation of rosettes, which represents the initial step of the phagocytic phenomenon in the cellular response of *H. polii* to the injection of sheep erythrocytes (Canicatti, 1988; Canicatti and Ancona, 1989).

A complement-like activity was recognized in invertebrates on the basis of functions analogous to those of the complement counterpart (Day *et al*., 1970; Anderson *et al*., 1972; Day *et al*., 1972; Laulan *et al*., 1983; Koch and Nielsen, 1984; Phipps *et al*., 1987). In the fall armyworm, a structural analogy was demonstrated between the major hemolymph protein and complement components (D’Cruz and Day, 1985). In Echinoderms (Bertheussen, 1983), the analogy to complement was deduced from the lytic action on rabbit erythrocytes that 'knowingly' activate the alternative pathway of the human complement system.
(Platts-Mills and Ishizaka, 1974). On the other hand, it was found that the purified
H. polii hemolytic molecule as well as the E. foetida, one can produce a lytic
effect on erythrocyte targets without the involvement of other proteins favored the
idea that a system which does not need an activating cascade, like complement,
exists in invertebrates (Canicatti, 1990). Study on such molecules in invertebrates
in recent times have been of immense interest, as these molecules take part in
important function of innate immunity. However, research works on molecules
with lytic nature that involve in immune functions are much limited especially in
large group of organisms like insects.

1.9 Insect model, Chrysomya megacephala

Among the most important groups of dipteran insects, the blowfly
Chrysomya megacephala (Fabricius, 1794) (Diptera: Calliphoridae) commonly
found in corpses has significant role in forensic, medical and veterinary sciences
(Greenberg, 1971; Goff and Odom, 1987; Goff et al., 1988; Goff, 1992; Centeno
et al., 2002; Gruner et al., 2007; Sukontason et al., 2007; Wang et al., 2008).
It is one of the species of synanthropic flies that have been reported to be
associated with unhygienic environment and involved in the propagation of human
teropathogens (Graczyk et al., 2001). It is a holometabolous insect and has three
larval instars found feeding on decomposing animal organic matter such as dead
animals or carrion. These decomposing organic matters contain abundance of
microorganisms. Consequently, the larvae have developed some defenses against
these pathogens, including rapid release of the antimicrobial molecules / proteins into their hemolymph (Faraldo et al., 2008). As they live in such highly contaminant habitats, the innate immune system in *C. megacephala* would definitely be better than any other insects.

### 1.10 Previous studies on *Chrysomya megacephala*

Few studies on antibacterial activity are already reported in the hemolymph and secretion from larvae and pupae of *C. megacephala* (Sahalan et al., 2007; Faraldo *et al.*., 2008; Mohamed, 2015; Ratcliffe *et al.*, 2015; Chaiwong *et al.*, 2016). But no attempt was made to isolate and purify these antibacterial (lytic) molecules. Hence, in the present study an attempt is made to detect, purify and characterize lytic molecule from the larval extract of *C. megacephala*.

### 1.11 Objectives of the present study

- Detection and preliminary characterization of hemolytic / lytic factor from the larval extract of blow fly, *Chrysomya megacephala*.

- Purification and molecular characterization of hemolytic molecule in the larval extract of *C. megacephala*.

- Functional analysis of purified protein for its antibacterial activity, especially bacteriolytic activity under *in vitro* conditions.