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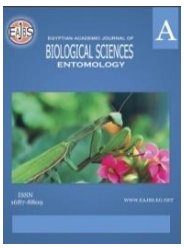

# Immune Responses and Bioactive Peptides of Insect Hemolymph

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## ABSTRACT

Hemolymph is an important defense line in insects. Hemolymph plays crucial defensive and immunological roles in insects. It includes hemocytes, the main immune cells, which perform various immunological functions such as phagocytosis, encapsulation, lysis of foreign bodies and release of humoral proteins. Two main immune responses that have been reported for insects are cellular and humoral defenses. Cellular immune responses are immediately carried out after an invasion of the hemocoel and include phagocytosis, nodulation and encapsulation. Insect hemolymph-derived peptides have a variety of therapeutic activities such as antimicrobial, anticancer and antiviral. The potential of these peptides to be promising effective agents for a panel of disease treatment makes them a main area of future research. The current review article explores some of the immune responses of insect hemolymph and the promising activities of their bioactive peptides.

## INTRODUCTION

The most important component of the circulatory system of arthropods is a fluid called hemolymph. Insect hemolymph has been recognized as the circulating fluid or blood of insects. Hemolymph plays crucial defensive and immunological roles in insects. Hemolymph is an important defense line in insects. It includes hemocytes, the main immune cells, which perform various immunological functions such as phagocytosis, encapsulation, lysis of foreign bodies and release of humoral factor, clotting proteins, and antimicrobial peptides. Hemocytes are functionally analogous to vertebrate leukocytes that perform various physiological and immunological functions in the body of arthropods. (Bulet *et al.,* 1999)

Insects are known as vectors of a wide range of animal and human parasites causing many diseases such as malaria, dengue, and yellow fever. Paradoxically, they are also a source of different natural constituents which can be employed in the development of natural bioactive compounds used for medical, veterinary and agricultural purposes (El-Tantawy, 2015). Insects have been widely used in traditional medicine in many parts of the world. In Chinese medicine, the beneficial effect of insects on different ailments has been known for over 3,000 years. Around 300 insect species are used in the invention of about

1,700 traditional Chinese medicaments (Ratcliffe *et al.,* 2011). Insect hemolymph has been recognized as a considerably rich natural source of unique therapeutic molecules complex fluid composed of hemocytes, plasma, and dissolved inorganic and organic compounds (carbohydrates, proteins and lipids) are the main constituents of hemolymph. (Sahalan *et al.,* 2007) .Lectin, hemocyanin, vitellogenin and hexamerins extracted from hemolymph of some insects participate in the host defense system and exhibited a wide range of therapeutic activities activity (Danty et al., 1998) (Barchuk et al., 2002; Varki et al., 2009) Insects have significant potential sources of antimicrobial peptides (AMPs) and the information on their antimicrobial effects is continuously increasing. These AMPs are released into the haemolymph where they attack elements of the bacterial or fungal cell wall. Four main groups of AMPs derived from insect hemolymph are cysteine-rich peptides, α-helical peptides, proline-rich peptides, and glycine-rich (Sahoo *et al.,* 2021).

Interestingly, the family of cecropins is the main group of insect hemolymph that possess anticancer and antiviral peptides derived *Musca domestica, Hyalophora cecropia,* and *Hyalophora cecropia* hemolymph. This review focuses on the different immune responses of insects and the bioactive peptides derived from insect hemolymph.

**Insect Immune Cells:**

The cells of insect hemolymph (hemocytes) play a main role in immunity. Prohemocytes are small rounded cells with large nuclei, which divide and may differentiate into plasmatocytes, granulocytes (granular cells), coagulocytes, spherulocytes and oenocytoids. Plasmatocytes contain lysosomal enzymes and they are the most abundant cell type, and participate, to some extent, in the synthesis of antimicrobial peptides during the humoral response. Plasmatocytes and granulocytes are the predominant phagocytic cells. Spherulocytes are oval or round cells with varying numbers of small spherical inclusions. Oenocytoids are large, binucleate, non-phagocytic cells that may contain prophenoloxidase. Coagulocytes have also been termed hyaline hemocytes and are involved in the clotting process. Other immune cells such as adipohemocytes which are characterized by the presence of fat droplets and lamellocytes play respective roles in encapsulation and melanisation of larger intruders (Kavanagh and Reeves, 2004).

**Immune Responses of Insects:**

The innate immune system of insects is subdivided into cellular and humoral defenses (Fig. 1). Cellular immunity refers to defense responses like phagocytosis and encapsulation that are mediated by blood cells (hemocytes). Humoral defenses in contrast refer to soluble effector molecules such as antimicrobial peptides, complement-like proteins, and the enzymatic cascades that regulate melanin formation and clotting.

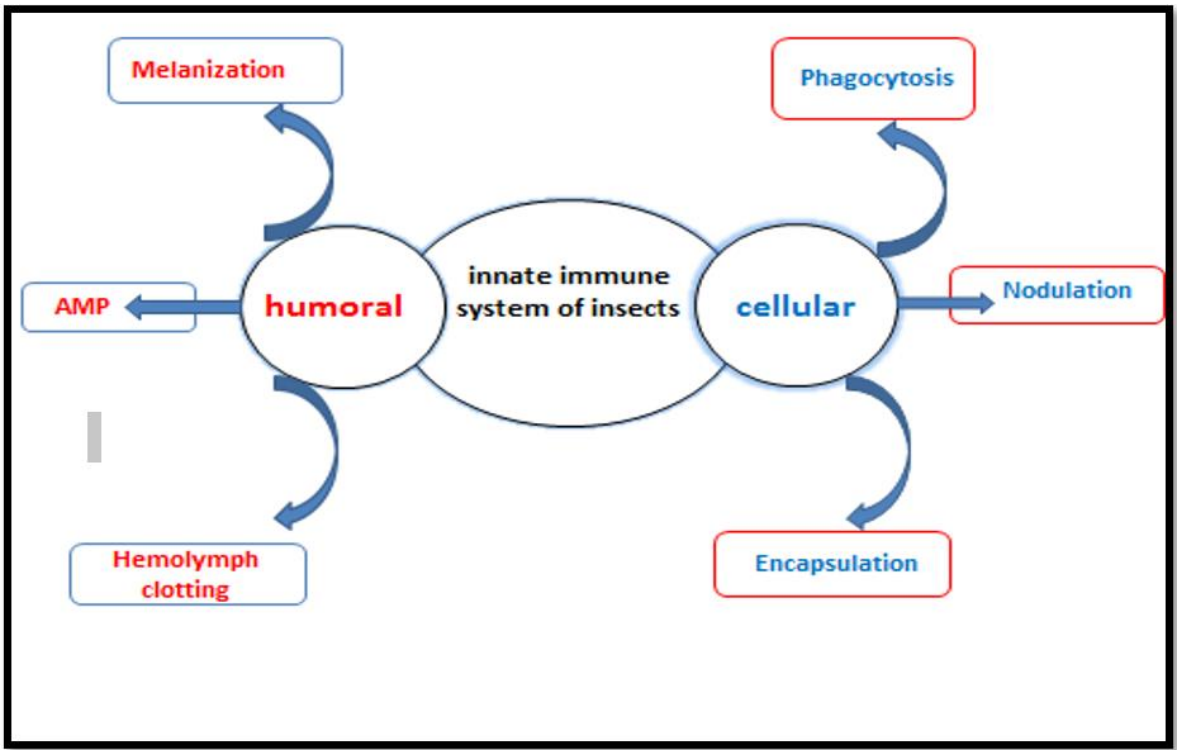


**Fig. 1**. The layout of the innate immune system of insects.

**Cellular Immune Responses of Insects:**

Cellular immune responses are immediately carried out after an invasion of the hemocoel and they are performed through hemocytes and include phagocytosis, nodulation and encapsulation.

**Phagocytosis:**

Phagocytosis is used for the destruction of small foreign organisms. Phagocytosis is initiated when a foreign object is recognized and bound by proteins in the plasma membrane of the phagocyte. The foreign object is then internalized into a membrane-delimited phagosome, the phagosome fuses with a lysosome, and hydrolytic enzymes digest the particle. Both granular cells and plasmatocytes are supposed to be primarily responsible for phagocytosis. The granulocytes of lepidopterans, hemipterans and mosquitoes, and the plasmatocytes of fruit flies have a diversity of targets including bacteria, yeast, apoptotic bodies, and abiotic particles like synthetic beads and India ink particles (Strand, 2008, Honti *et al.*, 2014, Hillyer, 2016).

**Nodulation:**

Hemocytes activate other mechanisms to control infections when the initial phagocytic immune response is not sufficient. To deal with large bacterial loads, in this immune process, granulocytes adhere to each other and form layers that surround dense bacterial aggregates. The granulocytes release their contents, which encase the bacteria in a flocculent material. In addition, the plasmatocytes aggregate around the surface of the nodule. Finally, the nodule is covered with layers of flattened hemocytes and it is melanized. Melanin-covered nodules efficiently isolate bacteria from the hemolymph (Carton *et al.*, 2002, Gandhe *et al.*, 2007).

**Encapsulation:**

Encapsulation is a cellular immune response used against large-sized pathogens such as protozoa, nematodes and eggs and larvae of parasitic insects. These virulent factors are encapsulated by being surrounded by layers of haemocytes where plasmatocytes are the effector cells of encapsulation. Plasmatocytes bind to the target in multiple cell layers until

they form a capsule around the invader. The capsule is normally melanized at the end by degranulation of crystal cells inside the capsule, the invading organism is killed by reactive cytotoxic products. This response is commonly employed by dipteran and lepidopteran larvae in response to infection with the eggs of parasitoid wasps. In Lepidoptera they used the plasmatocytes only while *Drosophila* for example used both plasmatocytes and lamellocyte for encapsulation (Hillyer, 2016, Rosales and Vonnie, 2017).

**Humoral Immune Responses of Insects:**

The humoral immunity response of insects consists of the processes of melanisation, haemolymph clotting and wound healing in response to injury and also involves the synthesis of a range of antimicrobial peptides (Kavanagh and Reeves, 2004).

**Melanization:**

Melanization is an enzymatic process of melanin formation. It is activated during wound healing and also in nodule and capsule formation against large pathogens or parasites in several insects, melanization is an immune effector mechanism involved in the killing of bacteria, fungi, protozoan parasites, nematode worms, and the eggs of parasitoid wasps. Melanization is phenotypically manifested as a darkened proteinaceous capsule that surrounds the invading pathogen, and the death of the pathogen presumably occurs via either oxidative damage or starvation, as the foreign agent becomes isolated from the nutrient-rich hemolymph (Nappi and Christensen, 2005, Cerenius *et al.*, 2008).

**Hemolymph Clotting:**

Insects have developed different mechanisms for the coagulation of hemolymph, in case of wounding, to prevent loss of body fluids, two types of clotting mechanisms have been identified. One of them was described in the cockroach (*Leucophaea maderae*) and the locust (*Locusta migratoria*) where the polymerisation of clottable proteins is catalysed by a $Ca^{2+}$dependent transglutaminase released from the hemocytes. The identified clottable proteins are lipophorin and vitellogenin-like proteins. The latter contains a region homologous to the 'D' domains of the von Willebrand factor tangentially involved in vertebrate blood clotting. The other type of coagulation has been best studied in horseshoe crab (Lymulus polyphemus), another arthropod, LPS and β-1,3-glucan trigger a serine protease chain reaction, finally leading to the coagulation of the hemolymph. In addition, serine protease activates the melanization cascade (Vilmos and Kurucz, 1998.

**Antimicrobial Peptides (AMPs):**

Although the cellular and humoral responses are effective in combating a microbial invasion, however, they are unable to completely clear the haemocoel if a large number of microorganisms invade. The last line of insect defense is the synthesis of a range of AMPs, which are released into the haemolymph when they attack the target. The principal site of synthesis is the fat body (tissue corresponding to the mammalian liver), also the hemocytes, the cuticular epithelial cells, the gut, the salivary gland, and the reproductive tract. AMPs are important parts of the innate immune system, they are small molecules that may present antibacterial, anticancer, antifungal, antiparasitic, and antiviral activity (Vilmos and Kurucz, 1998, Kavanagh and Reeves, 2004, Bulet and Stocklin, 2005, Guilhelmelli *et al.*, 2013)

Until now, more than 1,500 proteins with antimicrobial activity have been found and identified in different organisms such as plants, fungi, bacteria and animals; however, they are mainly present in insects. These small molecules have amino acid compositions ranging from 12 to 50 amino acids. The first insect AMP is the cecropin which was identified in 1980 from the pupae of *Hyalophora cecropia* (Strand, 2008, Jozefiak and Engberg, 2017).

The massive spread of infectious diseases showed a prominent resistance to conventional treatments and it has become an alarming phenomenon worldwide; Drug-

resistance phenomena involve not only antibacterial compounds, but also antiviral, antifungal, and antiprotozoal therapeutics. Currently, it estimates that drug-resistance cases result in 700,000 deaths per year (Organization, 2019). Therefore, there is a too urgent need to develop new therapeutics including AMPs. AMPs are naturally occurring peptides produced as a first line of defense against pathogenic infections by virtually all living species, from bacteria to mammals (Zhang, 2016).

Different studies have revealed that besides peptide charge (cationic or anionic), other characteristics such as size, primary sequence, conformation, structure, hydrophobicity, and amphipathicity could be essential for antimicrobial activity and mechanism of action of AMPs (Guilhelmelli *et al.*, 2013)

The Main mechanism of AMPs is to perturb the target cell membranes through the formation of ion channels or transmembrane pores. In this latter mode of action, AMPs destroy the bacterial cell by electrostatic forces, because most insect AMPs are cationic molecules, they penetrate the cell through the negatively charged particles present in the bacterial cell envelopes with which the peptide can interact (Yada et al., 2018). Other main targets for AMPs are lipids in bacterial cell membranes. In Gram-negative bacteria, the AMPs bind to anionic phospholipids and phosphate groups of lipopolysaccharides (LPS). While, in Gram-positive bacteria, the teichoic acid (anionic polymers) and lipoteichoic acids composing the peptidoglycan layer bind to AMPs (Hoskin and Ramamoorthy, 2008a)

There are four main models of membrane-pore formation, the first model is the barrel-stave model in which peptides bind to the cell membrane and insert into the hydrophobic core of the membrane, forming a pore and causing a leakage of cytoplasmic material and a decrease in membrane potential. In this way, membrane damage peptides, finally lead to the death of the cell. The second model is the toroidal-pore model where the peptides aggregate into lipid monolayers and form pores, finally leading to the destruction of the bacterial cell. The third model is the carpet model as high AMP concentrations are required to form micelle and destroy the microbial membrane. When the peptide concentration reaches the threshold, AMPs cover the membrane in clusters and cause the membrane to rupture in a surfactant-like manner. Finally, the fourth model is the aggregate model where the peptides and lipids are forced to form a peptide-lipid complex micelle. In this latter model, it is different from the carpet model, the channels formed by AMPs, lipids and water allow ions and intracellular contents to leak out, and then lead to cell death. These channels may also help AMPs transfer into the cytoplasm and exert function (Jozefiak and Engberg, 2017, Manniello *et al.*, 2021, Zhang *et al.*, 2021).

Insect AMPs can act also through non-membranolytic mechanism AMPs as they can penetrate into the bacterial cell without membrane break, causing bacterial death by interacting with intracellular targets by inhibiting DNA, RNA and protein synthesis, inhibiting protein folding, inhibiting enzyme activity and cell wall synthesis, and promoting the release of lyases to destroy cell structures (Brogden, 2005)

**Bioactive Peptides Derived from Insect Hemolymph:**

**Insect Hemolymph Antimicrobial Peptides:**

Basically, Basically, AMPs are found in insects based on their structure and amino acid composition they are four groups. They include cysteine-rich peptides (e.g., defensin and drosomycin), α-helical peptides (e.g., moricin and cecropin), proline-rich peptides (e.g., drosocin, apidaecin, and lebocin), and glycine-rich proteins (e.g., attacin and gloverin) (Sahoo *et al.*, 2021).

**Cysteine-Rich Antimicrobial Peptides:**

Defensins are a family of small, variable cationic arginine-rich peptides. Defensin peptides are ancient natural antibiotics with strong antimicrobial activity against a range of microorganisms. They consist of 18–45 amino acids with 6–8 conserved cysteine residues;

insect defensins contain 29–34 amino acids. The molecule of defensin is usually stabilized by three disulfide bonds and, aβ-hairpin is their major structural feature (Wu *et al.,* 2018).

Insect defensins are mainly active against bacteria such as *Micrococcus luteus, Aerococcus viridians, Bacillus megaterium, B. subtilis, B. thuringiensis*, and *Staphylococcus aureus.* Some insect defensins are also active against Gram-negative *Escherichia coli*. Insect defensins kill bacteria by forming channels in the bacterial cytoplasmic membrane (Yi *et al.,* 2014)

Hemolymph insect defensins are isolated from insect orders such as Diptera, defensin A, B and C derived from *Phormia terranovae, Aedes aegypti* and *Anopheles gambiae* as they showed potent antibacterial activity against Gram-positive bacteria and Gram-negative bacteria (Lambert *et al.,* 1989).(Lowenberger *et al.,* 1995, Vizioli *et al.,* 2001).

Moreover, Sapecin A and C-derived *Sarcophaga peregrine* have more effective against Gram-positive bacteria than against Gram-negative bacteria (Matsuyama and Natori, 1988). While the lucifensin which is isolated from *Lucilia sericata* is effective against Gram-positive bacteria only(Čeřovskỳ *et al.,* 2010).

Drosomycins are among the cysteine-rich antimicrobial peptides. Drosomycin was the first inducible antifungal peptide to be characterized in insects; drosomycin is a 44-residue peptide containing eight cysteine residues engaged in four intramolecular disulfide bridges. Drosomycin exhibits strong and selective activity against a relatively broad range of filamentous fungi (Fehlbaum *et al.,* 1994, Dimarcq *et al.,* 1998)

**α-Helical Antimicrobial Peptides:**

Cecropins are a family of cationic antimicrobial peptides including 31–39 residues. Cecropins (A, B and D) are the main insect consisting of 35–37 residues without cysteine. Cecropins can lyse bacterial cellular membranes and can also stop proline uptake as well as cause leaky membranes. Insect cecropins also have other names including bactericidin, lepidopteran, sarcotoxin, etc (Hultmark et al., 1982a) (Van Hofsten et al., 1985).

Cecropin A is an AMP with a stabilized α -helical structure, the accurate antibacterial mechanism of cecropin A is unclear, but there is primary evidence showing that the cell membrane is the target. Cecropin B is a naturally occurring linear cationic peptide consisting of 35 amino acids and is a member of the cecropin family with the highest antibacterial activity. Cecropin C is present in very low quantities; the antibacterial activity of cecropin C is rarely reported. Cecropin C is considered a precursor or degradation product of cecropin A . (Wu *et al.,* 2018).

The first isolated Cecropins AMPs were from the hemolymph of the lepidopteran *Hyalophora cecropia* and were characterized for their antimicrobial activity against several Gram-positive and negative bacteria. The order Diptera contains other three types of cecropin peptides as sarcotoxin peptide derived from the hemolymph of *Sarcophaga peregrina* (Flesh Fly) larvae and their activity against Gram-positive and negative bacteria (Okada and Natori, 1985). Cecropins peptides are also isolated from *Drosophila melanogasterit* with strong antifungal activity (Ekengren and Hultmark, 1999). In order lepidoptera, *Hyalophora cecropia* has cecropins A B, C, D, and E a with broad-spectrum activities against Gram-positive, negative bacteria and fungi. (Hultmark *et al.,* 1982b) .

α-helical antimicrobial peptides also include moricins. Moricins consist of a long alpha-helix with 8 turns from a 42 amino acid. The amphipathic N-terminal segment of the alpha-helix is mainly responsible for the increase in permeability of the bacterial membrane which kills the bacteria (Hemmi *et al.,* 2002). Moricins have activity against both Gram-negative and Gram-positive bacteria. Moricin was first isolated from the hemolymph of the silkworm *Bombyx mor.* The Moricins which are derived from *G.*

*mellonella moricins* also have potent activity against filamentous fungi and yeast. (Hara and Yamakawa, 1995 , Brown *et al.,* 2008).

**Proline-Rich Antimicrobial Peptides:**

Lebocins are consisting of 32 amino acids, which were first identified by Hara and Yamakawa (1995). Lebocin is a proline-rich and O-glycosylated peptide. In total, 41% of the amino acid sequence of lebocin is similar to abaecin, which leads to a major 34 amino acid antibacterial peptide in the honeybee *Apis mellifera*. Lebocins are active against Gram-negative and Gram-positive bacteria and some fungi (Casteels *et al.,* 1990, Hara and Yamakawa, 1995).

Apidaecins among proline-rich antimicrobial peptides. Apidaecins are small proline-rich peptides that have been identified in the orders of diptera, hymenoptera, hemiptera, and lepidoptera. They may not have high similarities in amino acid sequences, but they are all proline-rich and therefore should belong to the same family of proline-rich antimicrobial peptides. They are active against Gram-negative bacteria, Gram-positive bacteria, and some fungi. Apidaecins Ia, Ib, II In were identified from *Apis mellifera* and showed potent antibacterial activity against Gram-negative (Casteels *et al.,* 1989).

**Glycine-Rich Proteins Antimicrobial Peptides:**

Attacins are derived as pre-proportions containing a signal peptide, a pro-peptide (P domain), and an N-terminal attacin domain, followed by two glycine-rich domains G1 and G2 domains (SUN *et al.,* 1991, Hedengren *et al.,* 2000). They are a rather heterogeneous group of proteins, varying in size but rich in glycine residues (10–22%). Showed two groups derived from Attacins A–F based on their amino acid composition: attacins A–D are a basic group; and attacins E and F, which have acidic residues. Within each group, the forms are very similar. Attacins A–F are closely related antibacterial proteins, which are isolated from the hemolymph of immunized pupae of the cecropia moth *Hyalophora cecropia,* most attacins are active against E. coli and some selected Gram-negative bacteria (Hultmark *et al.,* 1983).

Gloverin is a basic, glycine-rich, and heat-stable antibacterial protein of ~14 kDa. Gloverin was isolated from the immune hemolymph of two lepidopteran species, the giant silk moth *Hyalophora gloveri* and the old-world bollworm *Helicoverpa armigera*. They are effective against Gram-negative bacteria and are inactive against Gram-positive bacteria, yeasts (Axen *et al.,* 1997, Mackintosh *et al.,* 1998).

**Insect Hemolymph Anticancer Peptides (ACPs):**

Most ACPs contain six cysteine residues forming three intramolecular disulfide bonds that assemble into hairpin-like α- helices, β-sheets, or mixed structures, but some extended structures have also been reported (Bulet and Stocklin, 2005b Hoskin and Ramamoorthy, 2008, Wang *et al.,* 2013).

The Cecropin family; cecropin A, cecropin B and cecropin XI derived from *Musca domestica, Hyalophora cecropia, Antheraea pernyi* and *Bombyx mori* showed potent antiproliferativeactivities against a panel of cancer cell lines such as HL-60, LS-174T, BEL-7402, HeLa, Hep2 and BGC823 cells (Cerón *et al.,* 2010, Jin et al., 2010, Xia *et al.,* 2013, Wu *et al.,* 2015). The *Lasioglossum laticeps* has 3 types of ACPs Lasioglossin LL-I, Lasioglossin LL-II and Lasioglossin LL-III with potent anticancer activities against PC12 cell (Chamorro et al., 2009). The CopA3 peptide derived from *Copris tripartitus* displayed antigrowth effects against human gastric cancer cells and Human leukemia cells (Kang *et al.,* 2012, Lee *et al.,* 2015).

Alloferon 1 and alloferon 2 peptides isolated from *Calliphora vicina* have shown anticancer activities against the P388 cell (Chernysh *et al.,* 2002). Moreover, pierisin-1 and pierisin-2 peptides were found in *Pieris rapae* and *Pieris brassicae* to have cytotoxic activity against human gastric carcinoma (Koyama *et al.,* 1996, Watanabe *et al.,* 1999,

Matsushima-Hibiya et al., 2000). Other ACPs such as DILRG-NH2 were derived from *Antheraea yamammai* with severe sytotoxic effects against rat hepatoma cells (dRLh84)(Yang *et al.,* 2004).

**Insect Hemolymph Antiviral Peptides (AVPs):**

Generally, analysis of the 280 AVP sequences in the database suggests that most AVPs are under 100 residues long, with an average length of 31 amino acids. AVPs commonly have strong net positive charges at physiological pH but are also largely amphipathic, with the average AVP sequence in our database displaying a +4 charge while being composed of nearly half hydrophobic residues. The AVPs in the database are likely biased to be membrane-disrupting peptides, as evidenced by their average Boman index of 1.24; additionally, 100 of the 280 unique peptides are predicted to generate an $\alpha$-helical structure (Wang *et al.,* 2016) (Lee *et al.,* 2022).

*Hyalophora cecropia* has cecropin A as AVP with potent antiviral activity against HIV; HSV-1 and 2; Junin virus. Also, the melitin derived from *Apis mellifera has* antiviral activity against HIV; HSV-1 and 2; Junin virus(Sample *et al.,* 2013, Hong *et al.,* 2014, El-Bitar *et al.,* 2015). Mastoparan 7 is an AVP derived from *Vespula lewisii* and it has potent cytotoxic activity against VSV; HSV-1; YFV RSV; WNV(Li *et al.,* 2011). Both AVPs Alloferon 1 and alloferon 2 were derived from *Calliphora vicina* with antiproliferative effects against influenza viruses A, B (Chernysh et al., 2002). The myristoylated peptide derived from *Heliothis virescens* displayed antiviral activity against HIV-1 and HSV-1 (Ourth *et al.,* 1994, Ourth, 2004).

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