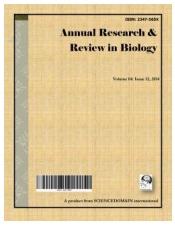

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Bioactive Compounds of the Salivary Glands from *Aedes aegypti* with Anti-Hemostatic Action

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MCM and NLLS designed the study. All authors managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

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ABSTRACT

Blood-sucking arthropods evolved a salivary cocktail of anti-hemostatic, platelet antiaggregant and vasodilators components that neutralize the effects of hemostasis and allow a successful blood supply occurs. In salivary glands from *Aedes aegypti* was found several components with anti-hemostatic action that inhibit the platelet aggregation and coagulation. These anticoagulants can prevent clot formation during the ingestion and digestion of blood meals by the *Ae. aegypti* insect. Thus, this review focused in *Ae. aegypti* saliva components that have anticlotting action and that has potential sources of novel pharmacologically active molecules, as potential therapeutic of new cardiovascular and anti-thrombotic drugs.

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Keywords: Aedes aegypti; salivary bioactive compounds; blood clotting; anti-hemostatic.

1. INTRODUCTION

Arthropod-borne diseases remain a major health problem worldwide in humans, such as malaria, dengue, Japanese encephalitis, yellow fever, and filariasis. It is estimated that there are approximately 14,000 species of arthropods that feed on blood [1]. Among the groups of blood-sucking insects, mosquitoes are given special attention, according to the World Health Organization (WHO), vectors of several emerging and reemerging diseases are responsible for over 1 million deaths/year. Of all mosquitoes families, the *Culicidae* family is the most important in terms of public health and contain genera *Aedes*, *Culex* and *Anopheles*. In this context, *Aedes* genus (e.g., *Aedes aegypti* and *Aedes albopictus*) in tropical areas, is known to be the primary vector of emerging and reemerging diseases such as yellow fever, dengue fever, ZIKV (Zika virus) and Chikungunya to humans, causing significant morbidity and mortality throughout the world [2-5].

Mosquitos *Ae. aegypti* females need blood to ensure the development of their eggs and to feed need to locate blood vessels of their vertebrate hosts. Thus, to be successful in blood meal, the mosquito *Ae. aegypti* female have two major obstacles: Hemostasis and the host immune system [6,7]. During blood feeding, this mosquito rapidly inject saliva into the host tissue, and this is important to the interaction between the parasite, vector, and mammalian host. In this cenarious, the haematophagous saliva exert a key function to the pathogen transmission, lead to blood coagulation and vasoconstriction at the site of vascular injury; prevent platelet aggregation, and stimulation the inflammatory and immune response from the host [8,9].

In the last years, several molecules from *Ae. aegypti* saliva were identified and has shown highly sophisticated pharmacological activities, for example, apyrase an enzyme that hydrolyses ADP, a nucleotide released by injured cells and a potent inducer of platelet aggregation [10]. Others major components in *Ae. aegypti* saliva with anticlotting and immunomodulator actions also were identified, such as tryptophan hydroxylase, antigen-5 family, D7 protein, salivary factor Xa-directed anticlotting, 30-kDa salivary allergen among others [11-14]. Thus, this review focused in *Ae. aegypti* saliva components that have anticlotting action, which may be potential sources of novel pharmacologically active molecules. In this regards, recent studies suggested that salivary components exhibit potential therapeutic application in clinical trials, such new cardiovascular and anti-thrombotic drugs and also may serve as vaccine targets against some diseases, including dengue, leishmaniasis and chagas disease.

2. TAXONOMY AND EVOLUTION OF *Aedes aegypti*

Arthropod families have at least fourteen members that contain more than 400 different genera and over 15,000 species, including *Ae. aegypti*, that belongs to the Kingdom Animalia, Phylum Arthropoda, Class Insecta, Order Diptera, Culicidae Family, Gender *Aedes*, Subgenus Stegomyia, Species *Aedes aegypti*. This species is the principal vector of some viruses worldwide, such as yellow fever, Zika virus, dengue and Chikungunya virus to humans, mainly because of its adaptability to urban life and its high susceptibility of the dengue virus [15-18].

The *Ae. aegypti* was originated in Africa [19] and was first described in Egypt, leading to the name [20]. It arrived in Brazil through the slave ships during the period slavery [21]. Ancestors of the domestic populations of *Ae. aegypti* lived on the sub-Saharan Africa, whose tended to breed in forested habitats and was predominantly zoophilic (blood meals in non-human animals. Today, this ancestral population still exists in forests and vegetated ecotones in sub-Saharan Africa [22] and is called by a subspecies *Aedes aegypti formosus*. After, two forms were identified as aegypti and formosus subspecies According to McClelland [23] morphologically, this ancestor is much darker than the adapted populations to human habitats, although this classification is quite variable.

The mosquito development for full transformation occurs, through the following stages: Egg, four larval instars, pupal and adult [24-29]. The salivary glands of adult mosquitoes are sexually dimorphic and it is clear that their structural and functional differences enable females to engage successfully in hematophagy The *Ae. aegypti* has anthropophilic habits and females perform hematophagy in daytime, with highest peak in

the period between 16 h and 18 h [28]. It has a quite imperceptible bite and can bite several people in order to acquire just one blood meal [30].

Males insect vector feed only sugar to sustain life, while the females can feed both sugar and blood, but the blood supply is very important to obtain the nutrients necessary to produce yolk proteins and eggs. During such blood meals, females can transmit pathogens to a vertebrate host. This is due the structural and functional differences between the salivary glands of adult insects, which are sexually dimorphic and enable females to carry out the biting the vertebrate host [31]. To locate a host, mosquitoes use a multi-sensory approach that includes detecting visual, olfactory, thermosensory, and gustatory cues to guide a series of behaviors collectively known as host seeking behavior [32,33]. Once a suitable host has been found, the mosquito lands and searches for a suitable site for the insertion of the mouthparts that lead an injury to the stratum corneum of the host skin. The skin is penetrated with active movements of the insect [34] and several seconds to minutes may pass until a suitable vessel or hemorrhagic pool is found, from where blood is sucked, resulting in to blood vessels and tissue injuries. This intradermal search for blood is known as probing time [1,35].

In this regards, the mosquito's saliva contributes to the ability of insect to locate the blood of vertebrates, preventing the hematoma formation generated by lacerations caused by the penetration of their mouthparts through of the host skin [36]. So in summary, the saliva in adult female *Ae. aegypti* is produced by secretory cells in the medial and lateral lobes of the salivary glands, and then it's released surrounding of the salivary duct that is connected to these extracellular secretory cavities of the mosquito [31].

To perform this process, the insect salivary glands suffered sophisticated evolving in its chemical constitution, secreting molecules with diverse enzymatic, that affect blood clotting, platelet aggregation, vascular contraction, host immunity, inflammation, and angiogenesis, among others [31,37]. These salivary products help in the acquisition of blood meals from vertebrate hosts, as well as for the digestion of sugar and nectar meals. In addition, they modulate vertebrate immune responses potentially increasing virus transmission, host susceptibility, viremia, disease progression and mortality [38-41]. Thus, recent studies confirm salivary compositional diversity from several hematophagous arthropods by transcriptome analysis, however, most of the identified proteins not yet had their known functions [42].

3. SOME SALIVARY COMPONENTS FROM *Aedes aegypti*

Over the course of several million years, the molecular diversity of saliva of bloodsucking insects may have arisen as a consequence of the evolutionary process that leads to insect adaptation to hematophagy. This evolution is associated with the expression of salivary active molecules from females insect vector that have a variety of pharmacological effects in order to maintain haemostasis, inflammation and adaptive immunity in the vertebrate host, based upon the release of saliva into the feeding site [37,40]. However, during blood meals, females also can transmit pathogens and the salivary constituents are important to infection maintenance and disease onset in the vertebrate host [9].

The past decade, several studies have focused on describing the sialome (set of RNA message + set of proteins found in salivary glands) for large-scale genomic, transcriptomic and proteomic analyses of salivary secretions of various blood-sucking insects, such as Anopheles [17,42-44]. Culex, Psorophora mosquitoes, Phlebotomine, Simulium and Aedes, [45,46]. These studies of sialotranscriptome analysis revealed a vast repertoire of vasodilators, anti-clotting and immunomodulator substances and enzymes, although, until now, many of these proteins families have not yet their defined functions [1].

Regarding *Ae. aegypti*, their salivary glands contain approximately 1–3 μg of protein, and a female mosquito injects about half of this protein during a single feeding. Thus, these insect's regurgitated constituents play an essential role in food ingestion, pathogens transmission and may affect vascular constriction, blood coagulation, platelet aggregation, inflammation, immunity and angiogenesis [38].

In this context, Ribeiro et al. [47] investigated the sialotranscriptome from *Ae. aegypti* mosquitoes, and they found in cDNA coding several components already described in other insects, as tryptophan hydroxylase, antigen-5 family, D7 protein, salivary factor Xa-directed anticlotting,

30-kDa salivary allergen, C-type lectin signature and related to the macrophage mannose receptor, a fibrinogen domain and related to vertebrate angiopoietins, salivary apyrase , vasodilator sialokinin, lyzozyme, gram-negative binding protein, serine proteases, calreticulin, bacterial adhesion proteins; mammalian testes protein, PAF-acetyl hydrolase, Adenosine deaminase, sphingomyelin phosphodiesterase; carboxylesterase; amylase; glucosidase, purine hydrolase among others. So far, other components have been described and their functions defined as shown in this review below.

Majority of these salivary molecules from *Ae. aegypti* are able of antagonizing the main effectors of immune responses and hemostatic responses of vertebrates due they represent a key obstacle for acquisition of the blood meal by hematophagous arthropods [1].

4. THE HEMOSTATIC SYSTEM AND SALIVARY COMPONENTS FROM *Aedes aegypti*

Blood is normally maintained in a fluid state, but upon tissue damage, or upon contact with a variety of extraneous substances can activate of hemostasis and this hemostatic system comprise a complex defense mechanism responsible for the control of blood loss resulting from a vascular injury. It is a regulated multifunctional process that involves multiple physiological cellular and acellular components, including the vascular response, platelet aggregation and the coagulation system. The Fig. 1 shows the coagulation cascade and its activation pathways, as described in detail below.

Hemostasis is categorized as either a primary or secondary process. Primary hemostasis involves the response of the vascular system and platelets to vessel injury. It takes place when there are injuries to small vessels during which the affected vessels contract to seal off the wound and platelets are mobilized, aggregate, and adhere to components of the subendothelium of the vasculature. Platelet adhesion requires the presence of various factors such as von Willebrand factor (vWF) and platelet receptors (IIb/IIIa and Ib/IX). Additional platelets are attracted to the site of injury by the release of platelet granular contents, such as adenosine diphosphate (ADP). The platelet plug is stabilized by interaction with fibrinogen. Secondary hemostasis involves the response of the coagulation system to vessel injury. It is required to control bleeding from large wounds and is a continuation of the primary hemostatic mechanisms. Whereas the outcome of primary hemostasis is the formation of the platelet plug, the outcome of secondary hemostasis is the formation of a thrombus [48].

The concept of coagulation cascade blood or secondary hemostasis, consists of a cascade of enzyme activation events in which serine proteases activate the proteins (pro-enzymes and pro-cofactors) in the next step of the cascade via limited proteolysis which was first proposed in 1964 [49]. This was described under the headings of the intrinsic pathway (dependent on contact activation by a negatively-charged surface, and involving coagulation factors XII, XI, IX, VIII and V), and the extrinsic pathway (dependent on tissue-factor being exposed to the circulation, and involving tissue factor and factor VII), converging on a common pathway to activate factor X, leading to conversion of prothrombin (factor II) to thrombin (factor IIa), culminating in the conversion of fibrinogen to fibrin (Fig. 1) [50]. Lastly, there is the polymerization of fibrin and the activation of platelets, leading to a blood clot. This process is protective, as it prevents excessive blood loss following injury (normalhemostasis) [43].

Disturbances of primary or secondary hemostasis could be associated with both hemorrhage and thromboembolic diseases [48]. Thus, the hemostasis equilibrium which is responsible for the maintenance of blood properties and keep it in its fluid form, is controlled by a complex system formed by interaction between cellular and protein phases of coagulation.

Extensive studies both experimentally and clinically is focused on isolating and characterizing highly specific anticoagulants from blood-feeding (haematophagous) animals, especially those targeting specific coagulation factors and using in antithrombotic drug design. Hirudin and tick anticoagulant peptide are examples of these substances that can be used in the treatment of pathological processes that affect the hemostatic system [51,52].

In this regards, the haematophagous insect's saliva has shown high clinical importance, since during the feeding process of insects occurs an injury in blood vessels and tissue, which in turn triggers the start of the hemostatic action of the host with the activation of the coagulation

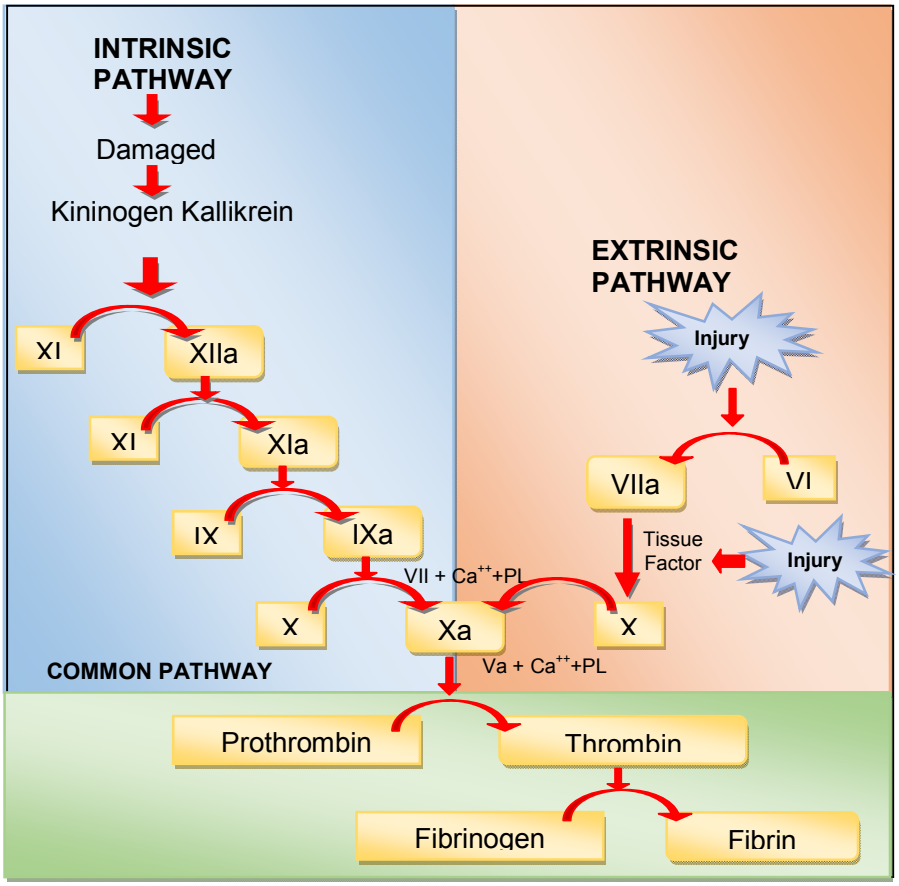

Fig. 1. The coagulation cascade consists of a series of serine proteases which activate each other sequentially. The intrinsic pathway is dependent on contact activation by negatively-charged surface, and involving coagulation factors XII, XI, IX, VII and V, while the extrinsic pathway is dependent on tissue-factor being exposed to the circulation and involving tissue factor and factor VII, converging on a common pathway to activate factor X, leading to conversion of Prothrombin to Thrombin and culminating in the conversion of fibrinogen to fibrin, which in turn polymerizes itself and, together with platelets, forms the blood clot *Source: Adapted from Adams and Bird (2009)*

cascade [37,53-55]. As the hemostasis is a redundant and complex system, these insects, including the *Ae. aegypti* evolved a mechanism to reverse this complexity through a salivary cocktail with anticoagulants, antiplatelet and vasodilators components that neutralize the effects of hemostasis and allow a successful blood supply occurs [13,15]. In this regards, in salivary glands from *Ae. aegypti* was found three general classes of anti-hemostatic agents that inhibit the platelet aggregation and coagulation, and/or induce vasodilation, as apyrase and D7 protein, inhibitors of platelet aggregation, the serpin, a serine protease inhibitor that acts on the factor Xa with anticoagulant activity, among others described below in detail. These anticoagulants target blood coagulation proteinases can prevent clot formation during the ingestion and digestion of blood meals by the *Ae. aegypti* insect. The Table 1 shows the shows the main action mechanisms of anti-hemostatic components found in vertebrates and invertebrates.

4.1 Apyrase

Apyrase was found in salivary glands of some insects to prevent blood clotting. It leads to platelet aggregation inhibition by the hydrolysis of adenosine triphosphate (ATP) and adenosine diphosphate (ADP), limiting their accumulation in the extracellular matrix, resulting in increase of adenosine monophosphate (AMP) and inorganic phosphate, which are unable to induce platelet

Table 1. Pharmacological properties exhibited by anti-hemostatic components

Components	Main action mechanisms	References
Apyrase	Leads to platelet aggregation inhibition by the hydrolysis of ATP and ADP resulting in increase of AMP and inorganic phosphate, which are unable to induce platelet aggregation and activate neutrophils. Removal of ATP and ADP limits their effect on platelet activation.	Clark, 2011; Ribeiro et al., 1984; Silva, 2009. Waidhet-Kouadio et al., 1998.
Serpin	Inhibition of factors IXA, XA and XIa; inhibition of thrombin.	Rosenberg, damus, 1973. Davie et al., 1991; Olson et al., 2010
D7 Protein	Binding to thromboxane a2; Inhibit the action of biogenic amines.	Arca et al., 2007; calvo et al., 2006, 2009.
Aegyptin	Inhibition of platelet aggregation and interaction with collagen.	Calvo et al., 2007. Andrews & berndt, 2004.
Kazal-type serine protease	Inhibitor of thrombin, it has two important regulatory regions besides the active site, exosites 1 and 2, which are binding sites for fibrinogen and heparin, respectively, and that is a key in blood coagulation.	Watanabe et al., 2011.

aggregation and activate neutrophils [36,56-58]. In summary, when insect feeding, ATP and ADP are released from damaged cells and activated platelets, thus they stimulate platelet aggregation and mast cells degranulation at the bite site. Then, removal of ATP and ADP by salivary apyrase reduces the pain caused by these extracellular nucleotides and limits their effect on platelet activation [44,59].

The apyrase has been detected in mosquitoes [60], stink bugs [61], blackfly [62], ticks [63,64], fleas; and *Culicoides* [65], and is synthesized in the salivary gland of female adults of *Ae. aegypti* and accumulates on the distal side lobes [60]. For molecular cloning and sequence analysis were revealed at least three classes of apyrases of different evolutionary origin. They are represented by the apyrases of the yellow fever mosquito *Ae. aegypti* [11,66] the intracellular parasite *Toxoplasma gondii* [67], and the bedbug *C. lectularius* (Valenzuela, 1998). The *T. gondii* apyrase belongs to a large family of ecto-ATPases that are found in a wide variety of organisms and tissues ranging from plants [68] to humans [69]. The *C. lectularius* apyrase belongs to a novel type of ATPases [70], and *Ae. aegypti* apyrase shows a high degree of sequence similarity to 5'-nucleotidases from different organisms [11].

Ae. aegypti apyrase (ATP diphosphohydrolase) is an enzyme member of the family 5'-nucleotidase with 68-kDa that inhibits platelet aggregation and prevents activation of neutrophils [11,60,71,72]. *Ae. aegypti* apyrases are different of others apyrases, such as apyrase of 37.5-kDa from *Cimex lectularius* belongs to a novel protein family showing significant similarity to phlebotomine apyrases [61,70,73] and to human and to rat apyrases [74,75]. The 5'-nucleotidase apyrase from *Ae. aegypti* works with either Ca^{2+} or Mg^{2+}, and the pH optimum is about 9 (although the enzyme is still very active at physiological pH) [61,76].

4.2 Serpin

Serpins are a proteins superfamily originally grouped together as serine protease inhibitors, which all of the endogenous thrombin inhibitors are members to this superfamily and their action mechanism are common to most members [77,78]. Structurally, serpins can contain 350 to 400 amino acids, a molecular weight of 40 to 55 kDa and consist of a mixed α/β fold [79] The serpins structure is characterized by three β-sheets (A, B and C) and eight or nine α-helices and the "reactive center loop" (RCL) [78]. The RCL is a protein motif of 20 amino acids, located near the C-terminus of the protein, which is the most important region for serpins inhibitory activity. This motif contains a scissile bond between the so-called residues P1 (the N-terminal portion of the cleavage event) and P1 '(C-terminal portion of the cleavage event) which is cleaved by the target protease. This cleavage triggers structural rearrangement of both the protease and the inhibitor in a suicide mechanism that irreversibly complexes and

inactivates both interacting partners [80,81]. The mechanism involves the attack of protease in the portion P1 and P1' of the serpin, can be which can lead to conformational change and formation of a serpin-enzyme complex inactive covalently bonded [82]. It can also follow another route where a protease cleaves the serpin making it inactive protease maintains its activity [78,83].

Most serpins are inhibitors of chymotrypsin like serine proteases, although have been identified additional cross-class serpin inhibitors and inhibit cysteine protease family members such as the caspases and cathepsins [84]. In addition, there are members of this superfamily that are lacked of any proteinase inhibitory properties and serve other functions, such angiotensinogen, ovalbumin, transporters or chaperones [77,78,85]. So far several sequences of serpin were identified (over 1500) in the genomes of all organisms of life, including nematodes, virus, insects, higher plants and vertebrates and 36 confirmed human serpins and were classified in 16 clades (designated A through P) [78,86]. In human, the antithrombin is a main plasma serpin that is involved in control and regulation of coagulation, inhibiting the thrombin and clotting factors IXa, Xa and Xia [87]. This serpin circulates in the blood in a "repressed" with low capacity to prevent the formation of clot [48,86]. In addition, others serpins are involved in the clotting process, such as heparin cofactor II [88], (α-2 macroglobulin [89], Protein Ca [90] and the α1-antripsina [91].

In hematophagous insects, the serpins are found in their salivary glands, being involved in a wide variety of physiological processes, including in the modulation of immune response, coagulation, fibrinolysis, complement regulation and inflammation or angiogenesis [78,92,93]. Regarding *Ae. aegypti,* Stark and James [15] found a serine protease inhibitor-like molecule of 56 kDa, in salivary extract from female insect, which inhibited both intrinsic and extrinsic coagulation pathways by inhibition specifically coagulation factor Xa. The biochemical characterization of the FXa-directed anticoagulant revealed a reversible, non-competitive and non-covalent kinetic, with no activity against thrombin and limited activity against trypsin. Furthermore, these authors reported that there are about 0.2–2 ng of this anticoagulant in each 1 mg of total protein of salivary extract. They were based on the similarity in molecular masses of FXa (46 kDa) and AFXa (56 kDa), which is roughly consistent with a 1:1 ratio of inhibitor to enzyme expected for a physiological inhibitor [15]. In 1998, these same authors isolated and characterized a gene designated anticoagulant factor Xa (AFXa), encoding of this novel Factor Xa-directed anticoagulant of salivary glands from female *Ae. aegypti*. From molecular blast analysis of the AFXa conceptual translational product was shown a highest degree of amino acid sequence identities and similarities with serpin-like serine protease inhibitors, such arginine-serpin, plasminogen activator inhibitor-2, from human, mouse, and rat [13].

4.3 D7 Protein

The D7 protein is a most abundant secreted protein in the salivary glands of female hematophagous arthropods [94-96]. Valenzuela et al. [96] suggested that D7 proteins should be between 10% and 50% of the salivary protein, varying depending on the insect vector. In the case of mosquitos and sand flies salivary glands is approximately 1-3 μg of protein content and half of this protein is discharged during a blood meal [60,97,98].

These proteins, known as multifunctional molecule, are related to the odorant-binding protein (OBP), which is adapted to bind small ligands, such as host biogenic amines (serotonin, histamine and norepinephrine) that may antagonize vasoconstriction, platelet aggregation and pain [42,45]. Thus, the mosquito D7 protein, during feeding, acts as an anti-hemostatic factor, antagonizing the vasoconstriction and platelet aggregation, plays an important role in facilitating blood-feeding process and indirectly may improve the pathogens transmission [42,96,99].

The first D7 gene encoding was reported 15 years ago, in the salivary glands from mosquito *Ae. aegypti* [12]. In this regards, Valenzuela et al. [96] reported the proteins expressed in the salivary glands and among the 31 novel protein sequences are 4 additional members of the D7 protein family (1 new D7 protein member and 3 short D7 protein). Then, the most abundant salivary cDNA coded for protein sequence having high similarity to the D7 protein family. This new D7 protein is named D7Bclu1. Another cDNA coding for a truncated member of the D7 protein family was D7Cclu23. They, also, described 2 new short D7 proteins that are similar to D7Cclu23, but have no similarities to other proteins in the NR database.

In addition, the D7 protein exists in two forms (along and short) in the mosquito genome, which contain one and two OBP-like domains, respectively [96,100,101]. The along form (30-35 kDa) is found exclusively in mosquitoes and sand flies and the short form (~15 kDa) is found in other insects [102,103]. Regarding to *Ae. aegypti*, Juhn et al. [31] reported the in situ hybridization patterns of 30 genes expressed in the salivary glands of adult female, of these three members were of the D7 gene family, one short isoform D7s2 (AAEL 006423) localized only in the distal-lateral lobes, and two long isoforms, D7L1 (AAEL006417) and D7L2 (AAEL006424), found in distal-lateral and medial lobes. Their findings support the argument that these genes encodes proteins play a role in binding agonists of haemostasis, inhibiting the vasoconstriction and platelet aggregation, while promoting blood-feeding [31,42].

Further studies also reported that D7 proteins could perform other functions unrelated to binding of small ligands, such as one short D7 protein from *Anopheles stephensi*, named hamadarin, which showed to prevent kallikrein activation by Factor XIIa [104]. Alvarenga et al. [59] showed the D7 family protein functions (D7L1 AnSt) that bind to thromboxane A2, thus acting to inhibit platelet activation during feeding of Anopheles mosquitoes. Moreover, Aedes D7 protein long fought also efficiently norepinephrine contraction in rat aortic rings, and it has been shown that the N-terminal domain of AeD7 binds with high affinity to cysteinyl leukotrienes, which act as inflammatory mediators [42,105].

4.4 Aegyptin

Aegyptin is a 30 kDa mosquito salivary gland protein that has anti-hemostatic effect and is an allergen, thus facilitating the blood-feeding process and also indirectly improves the pathogens transmission [103]. It binds to specific platelet glycoprotein VI (GP VI), integrin α2β1, von Willebrand factor (vWF) and collagen (KD 6.0 nM) and inhibits platelet aggregation [106]. In addition, aegyptin attenuates platelet adhesion to either soluble or fibrillar collagen and inhibits vWF interaction with collagen under static and high-shear conditions. In addition, aegyptin acts as a specific ligand for collagen and inhibits platelet activation and thrombocyte aggregation. In this regards, the collagen is a matrix protein that plays a pivotal role in the process of primary hemostasis, initiates recruitment of circulating platelets and triggers platelet activation cascade, which triggers and stimulates thrombin formation [17,103]. In this regards, the aegyptin blocks GPVI interaction with collagen and inhibits platelet aggregation and adhesion [17]. Surface plasmon resonance identified a high-affinity interaction between RGQOGVMGF (where O is hydroxyproline), a peptide corresponding to the collagen-binding site for vWF, and aegyptin [41]. Aegyptin also recognizes the peptides (GPO) and GFOGER with low affinity (micromolar range), which represent the glycoprotein VI- and integrin alpha2beta1-binding sites on collagen, respectively [41].

Aegyptin is a protein commonly found in sialotranscriptomes of mosquitoes and black flies [47], including Culex sp, and Anopheles sp [107], Aedes allergen. In addition, Aegyptin was first identified as 30-kDa in Aedes allergen [14,108]. In this regards, Calvo et al. [17] reported that *Ae aegypti* salivary gland expresses aegyptin, a potent collagen-binding protein that prevents its interaction with three major ligands, namely, GPVI, vWF and integrin α2β1. These authors showed that aegyptin binds to soluble collagen I-III, but no interaction was observed with other matrix proteins including laminin, vitronectin, fibronectin, vWf, and fibrinogen. Juhn et al. [31] identified a aegyptin gene (AAEL010235), this gene is accumulate only in the cells of the distal-lateral lobes, except for the transcripts of aegyptin, which also accumulate in the intermediate region and distal tip of the proximal-lateral lobes These It was found that aegyptin recognizes with high affinity the sequence involved in collagen interaction with vWF, and also interacts with GPVI and integrin α2β1 binding sites. Aegyptin effectively inhibits carotid thrombus formation *in vivo*. In *Ae. aegypti* salivary gland, Calvo et al. [17] also identified as a high-affinity binding site for aegyptin, the senquence RGQOGVMGF (O is hydroxyproline) that mediates collagen interaction with von Willebrand Factor (vWF). However, the aegyptin recognizes with low affinity the peptides (GPO) and GFOGER, representing the glycoprotein VI and integrin α2β1 binding sites, respectively, that binds in collagen and prevents platelet adhesion and aggregation. In addition, in vivo model, these authors showed that the aegyptin prevents laser-induced carotid thrombus formation. Other study also showed that doses of 100 µg/kg of aegyptin displays effective anti-thrombotic activity in rats, suggesting that aegyptin is a suitable molecule to inhibit platelet-collagen interaction *in vivo* [109]. Similarly, aegyptin from *A. stephensi* and *Ae. aegypti* salivary glands also inhibit platelet

aggregation by interfering with collagen recognition [16].

Regarding, vectors like *Anopheles stephensi* and *Simulim nigrimanum* also express salivary collagen-binding proteins that prevent collagen-induced platelet aggregation were the exposure of collagen in damage to the endothelium plays an important role in the early stages of the hemostatic plug formation following vascular injury [109,110]. Aegyptin displays sequence and functional similarities to AAPP, a collagen-binding protein from the salivary gland of *Anopheles stephensi* [111].

4.5 Kazal-type Serine Protease

Kazal type serine protease inhibitors (KPIs) is one of the thrombin inhibitors, which contain one or more Kazal inhibitory domains linked together by peptide spacers of variable length [112]. In Kazal domain is found six well-conserved cysteine residues capable of forming three intra-domain disulfide bridges between cysteine numbers, C1:C5, C2:C4, C3:C6, resulting in a characteristic three-dimensional structure [41,113]. The kazal-type domains are composed of 40-60 amino acid residues including some spacer amino acids and in tertiary structure includes one α-helix and one anti-parallel β-sheet [112,114]. The α-helix is surrounded by an adjacent three stranded β-sheet and loops of peptide segments [112]. Some amino acid residues in the Kazal motif are relatively conserved, but most of them are quite variable both within and among the invertebrate species [115]. In this regards, the specificity within Kazal-type inhibitors is determined for predicted reactive site, P1 amino acid residue, which is located at position C2-X-P1. Whereas, outside of the conserved cysteine residues, there are high amounts of variability in other amino acid residues [112].

The KPIs are grouped into the family I1 of the serine protease inhibitors that have been reported since 1980's [116]. This family was named by Kazal et al. [117], who were the first to isolate a pancreatic secretory trypsin inhibitor (also known as SPINK1). The inhibition mechanism of the Kazal proteinase inhibitory domain is due each Kazal domain acts as a substrate analogue that stoichiometrically binds competitively through its reactive site loop to the active site of cognate proteinase forming a relatively stable proteinase–proteinase inhibitor complex [112].

The first Kazal-type thrombin inhibitor in a haematophagous insect was identified by Friedrich et al. [118] that reported a double headed Kazal-type thrombin inhibitor, rhodniin, from *Rhodnius prolixus*. In invertebrates, a large number of proteins containing Kazal-type domains have been identified in many blood-feeding arthropods including in mosquitoes, ticks, triatomines and flies [110,119,120,121]. These insect Kazal-type inhibitors are known to inhibit thrombin, chymotrypsin, trypsin, plasmin, factor XIIa, subtilisin A and elastase [118,120,121].

Examples are the thrombin inhibitors, rhodniin, infestin and dipetalogastin, isolated from blood-sucking insects, *Rhodnius prolixus, Triatoma infestans* and *Dipetalogaster maximus*, respectively [122-124]. Others examples is a tryptase inhibitor, LDTI (Leech Derived Tryptase Inhibitor) [125], subtilisin inhibitor, infestin 1R [123], and elastase inhibitor, CmPI-II [126]. These inhibition of the coagulation cascade to facilitate fluidity in the mouth parts and midgut following blood-feeding on a host [110,127].

Regarding to *Ae aegypti*, Ribeiro et al. [44] analyzed a set of 3776 Salivary Gland cDNA sequences and indentified 573 new transcripts of putative secretory proteins from *Ae. aegypti.* Among those sequences, these authors found Kazal-type putative protease inhibitors, including the sequence gij94468720, which was expressed in salivary glands and in carcass of female and also in whole male. In addition, Watanabe et al., [127] expressed, purified and characterized for first time a putative Kazal-type serine protease inhibitor that is present in different tissues of *Ae. aegypti*, which was named *Ae. aegypti* Trypsin Inhibitor (AaTI). In addition, these authors cloning, expression, purification and characterization a recombinant AaTI (rAaTI), and for multiple alignment of AaTI amino acid sequence with other Kazal-type inhibitors revealed high similarity to non-classical Kazal-type inhibitors such as dipetalogastin [124], infestins [120,122], LDTI [125], brasiliensin [128], and a Kazal-type inhibitor from *L. vannamei*. These authors also showed that the AaTI can act as anticoagulant during the feeding and digestive processes for inhibition mechanism for thrombin and trypsin from different development stages of *Ae. aegypti*. Posteriorly, Watanabe et al. [129] showed that the rAaTI was able to prolong prothrombin time, activated partial thromboplastin time and thrombin time. In addition, the rAaTI contains a C-terminal charged

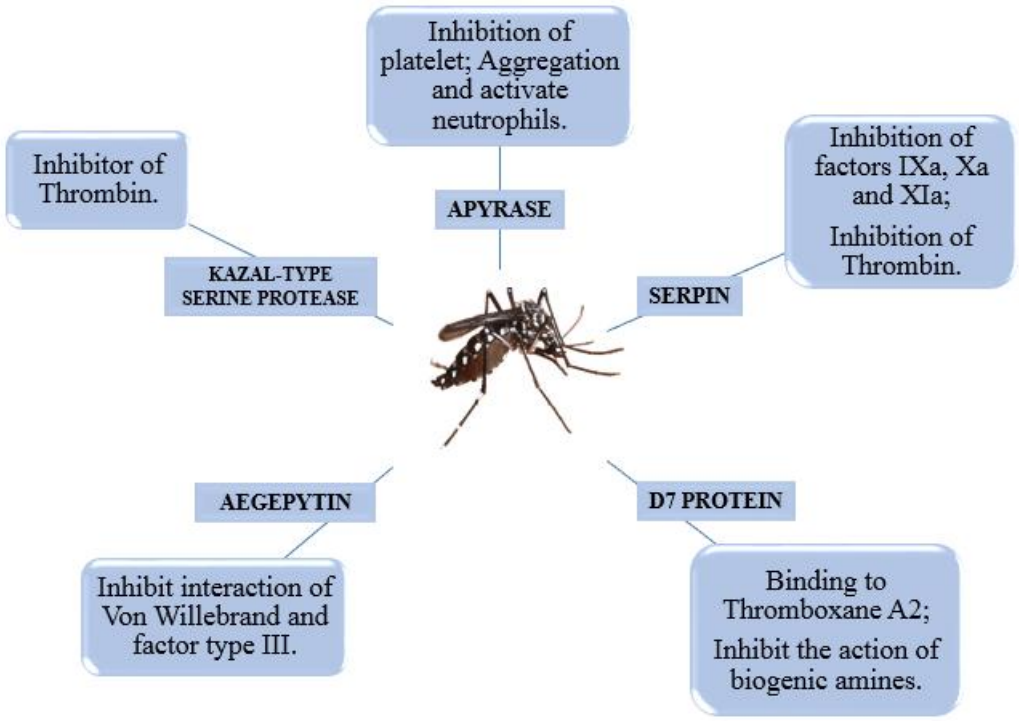

Fig. 2. Anti-hemostatic components of salivary gland from Aedes aegypti. In summary, the apyrase, aegepytin and D7 protein inhibit the primary hemostasis, serpin and kazal-type serine protease inhibit the coagulation cascade (secondary hemostasis)

Source: Own authors

peptide, the same as for other thrombin inhibitors, like hirudin [130] dipetalogastin [124] and rhodniin [131] suggesting that C-terminal region might be important to the rAaTI inhibits the thrombin by interacting with thrombin exosite 2 [127]. Then, these authors showed that the rAaTI may bind to the same region where antithrombin III or heparin binds on the thrombin surface. Classical inhibition experiments showed an uncompetitive inhibition mechanism for rAaTI and thrombin [127]. In this regards, thrombin is a serine protease, being a key enzyme of the blood coagulation cascade and also an important platelet aggregation activator, that has two important regulatory regions besides the active site, exosites 1 and 2, which are binding sites for fibrinogen and heparin, respectively [110,129]. The Fig. 2 above displays a summary of the main effects of the anti-hemostatic components of salivary gland from *Aedes Aegypti*.

5. CONCLUSION

From these findings, it was possible to prove that in order to facilitate their blood meals, the blood-sucking arthropods, such as *Ae. aegypti* have elaborated a wide range of the salivary components with anticoagulant action that plays an essential role in host hemostatic defense, facilitating the maintenance of the blood flow from the feeding site to the insect digestive tract.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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