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Analysis of Anticoagulant Proteins from Some Haematophagous Insects of Bangladesh

Khalid-Bin-Ferdaus, Khandaker Md.

University of Rajshahi

M.Phil
Thesis

ANALYSIS OF ANTICOAGULANT PROTEINS FROM SOME HAEMATOPHAGOUS INSECTS OF BANGLADESH

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A Thesis submitted to the Department of Genetic Engineering & Biotechnology, University of Rajshahi, Rajshahi, in partial fulfillment of the requirement for the Degree of Master of Philosophy

SUBMITTED BY
KHANDAKER MD. KHALID-BIN-FERDAUS
Examination Roll No.: 14
Registration No. 2972
Session: 2010-2011

June,
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DEPARTMENT OF GENETIC ENGINEERING & BIOTECHNOLOGY
UNIVERSITY OF RAJSHAHI
RAJSHAHI-6205, BANGLADESH

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DEDICATED TO MY BELOVED PARENTS AND TEACHERS WHO GAVE ME THE LIGHT OF KNOWLEDGE

DECLARATION

I hereby declare that the work embodied in this M. Phil thesis has been carried out in the Department of Genetic Engineering and Biotechnology, University of Rajshahi, is the result of my own investigation.

Khandaker Md. Khalid-Bin-Ferdaus

CERTIFICATE

This is to certify that the thesis entitled "Analysis of anticoagulant proteins from some haematophagous insects of Bangladesh" has been submitted in fulfillment for the requirements of the degree of M. Phil in the Department of Genetic Engineering & Biotechnology, University of Rajshahi, Bangladesh.

This is further certified that the research work embodied in this thesis is original and have not been submitted before in substance for any other degree or diploma of this University.

Prof. Dr. Md. Abu Reza
Supervisor

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The author

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CHAPTER 1

INTRODUCTION

Anticoagulant is treated as the first line of defense against the consequences of a blood clot, including deep vein thrombosis (DVT), stroke arising from atrial fibrillation (AF), pulmonary embolism (PE) and acute coronary syndrome (ACS). Medications have been used to prevent these fatal blood clots from the very beginning. However, for centuries, research work had been carried out to find novel anticoagulants to solve the problem of thrombotic disorder.

Hematophagous (from the Greek words αἷμα haima “blood” and φάγειν phagein “to eat”) insects contain multiple biologically active compounds such as cement, vasodilators, platelet aggregation inhibitors, anticoagulants, prostaglandins, and allergens in their saliva and they insert those to the host to create an optimal environment during their prolonged feeding (Ribeiro, 1987; Jaworski *et al*., 1990; Wang *et al*., 1996; Zhu *et al*., 1997 and Mans *et al*., 2004). In absence of anticoagulant components in the saliva blood will clot inside insects feeding tube and they will starve to death. So, anticoagulants in their saliva keep blood in liquid form. These anticoagulant proteins have remarkable biopharmaceutical applications in treatment of various thrombotic disorders. Therefore, these insects and their salivary gland is a virtual gold mine for drug lead compounds for future anticoagulant drug development.

Numerous anticoagulants have been isolated from the saliva of the blood sucking animals which they use during their feeding (Basanova *et al*., 2002 and Zavalova *et al*., 2002). The most famous anticoagulant protein is ‘hirudin’ which was isolated from leech. It is used as a commercial drug for thrombosis now a day (Markwardt, 2002). Another type of anticoagulant protein is ‘variegin’ that is recently discovered from the bont tick of Slovania (Koh *et al*., 2007). Apart from hirudin (Fig.-1) and variegin (Fig.-2) there are also many kinds of anticoagulant non proteinous substances identified from different insect species (Mans*et al*., 2004 and Mulenga, 2000) and scientists still try to develop novel anticoagulants from different sources.

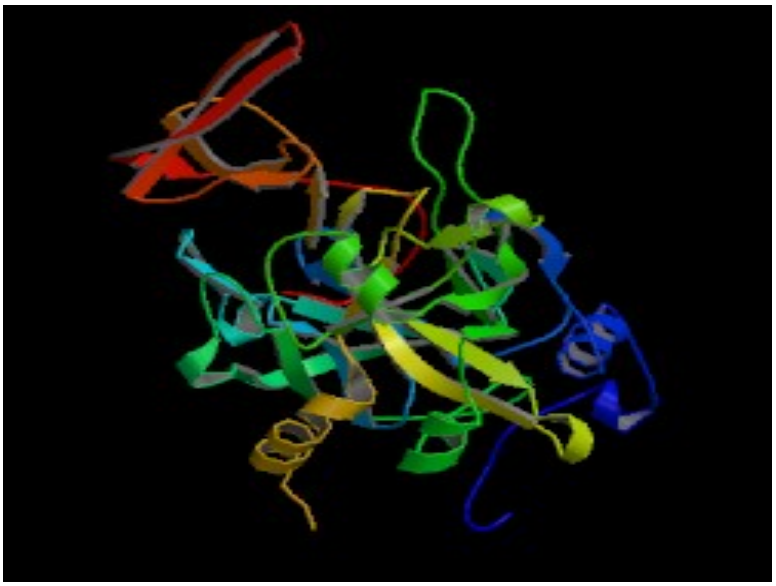


Fig.-1: 3D structure of hirudin

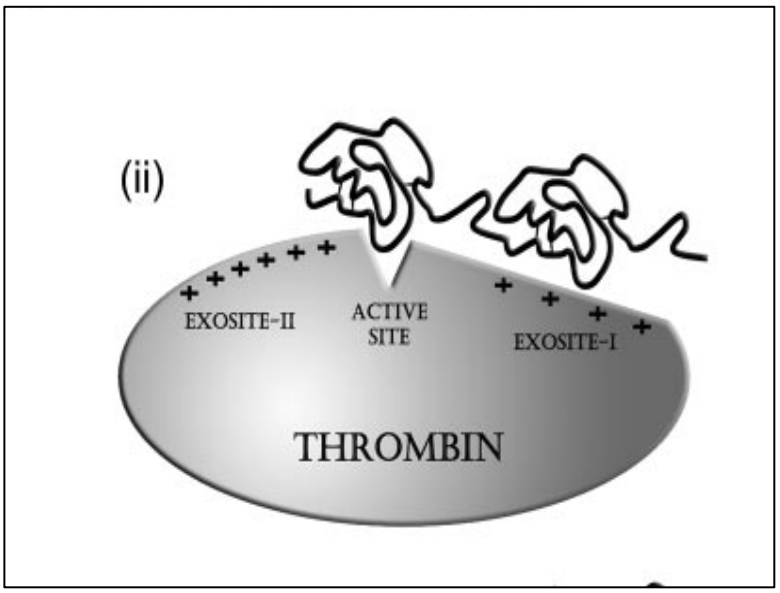


Fig.-2: 3D structure of variegin

1.1 Blood Coagulation

As the current thesis deals with anticoagulant proteins which is directly related to blood coagulation, it would be nice to first discuss about the blood coagulation process. Blood coagulation is a complex process by which the blood forms clots to block and then heal a lesion and stop the bleeding. It is a crucial part of hemostasis where a damaged blood vessel wall is plugged by platelet and a fibrin-containing clot to stop the bleeding, so that the damage can be repaired.

Precise control on coagulation is crucial for survival. Because, failure to clot blood at proper time can be fatal as seen in case of hemophilia, on the other hand unwanted clot formation is also extremely dangerous as seen in case of Disseminated Intravascular Coagulopathy (DIC) or stroke.

When damage occurs to a blood vessel, circulating platelets immediately get trapped at the injury site. On accumulating the platelets 'plug' in the leakage site platelets provide the first step damage control (primary hemostasis). This mechanism is supplemented by 'blood coagulation' (secondary hemostasis), which is the most important means of defence against bleeding.

The coagulation is a series of reactions, in which a number of zymogen (coagulation factor) of serine protease enzymes and its glycoprotein co-factors are activated by proteolytic cleavages. Each activated protein catalyzes the next reaction in the cascade. The cascade functions as a series of positive and negative feedback loops that control the activation process. The ultimate goal of the pathway is to produce thrombin, which can then convert soluble fibrinogen into fibrin that forms a clot. This process is controlled by a signaling cascade consisting of 13 coagulation factors from I to XIII which interact and activate each other (Hoffman*et al*., 2005 and Johne *et al*., 2006).

The coagulation process (Fig.-3) has three pathways that lead to fibrin formation-

1. The contact activation pathway (the intrinsic pathway)
2. The tissue factor pathway (the extrinsic pathway)and
3. The final common pathway

1.1.1 Contact activation pathway (intrinsic)

The contact activation pathway begins with formation of the primary complex on collagen by high-molecular weight kininogen (HMWK), prekallikrein, and FXII (Hageman factor). Prekallikrein is converted to kallikrein and FXII becomes FXIIa. FXIIa converts FXI into FXIa. Factor XIa activates FIX, which with its co-factor FVIIIa form the tenase complex, which activates FX to FXa (Fig.-3).

1.1.2 Tissue factor pathway (extrinsic)

The main role of the tissue factor pathway is to generate a "thrombin burst", a process by which thrombin is released instantaneously. FVIIa circulates in a higher amount than any other activated coagulation factor. So when damage occure to the blood vessel, endothelium Tissue Factor (TF) is released, forming a complex with FVII and in so doing, activating it (TF-FVIIa). TF-FVIIa activates FIX and FX. FVII is itself activated by thrombin, FXIa, plasmin, FXII and FXa. The activation of FXa by TF-FVIIa is almost immediately inhibited by tissue factor pathway inhibitor (TFPI). FXa and its co-factor FVa form the prothrombinase complex which activates prothrombin to thrombin (Fig.-4). Thrombin then activates other components of the coagulation cascade, including FV and FVIII (which activates FXI, which in turn activates FIX), and activates and releases FVIII from being bound to vWF. FVIIIa is the co-factor of FIXa and together they form the "tenase" complex which activates FX and so the cycle continues.

1.1.3 Final common pathway

Thrombin has a large array of functions. Its primary role is the conversion of fibrinogen to fibrin (Fig.-5), the building block of a hemostatic plug. In addition, it activates Factors VIII and V and their inhibitor activated protein C (APC, in the presence of thrombomodulin), and it activates Factor XIII, which forms covalent bonds that crosslink the fibrin polymers that form from activated monomers. Following activation by the contact factor or tissue factor pathways the coagulation cascade is maintained in a prothrombotic state by the continued activation of FVIII and FIX to form the tenase complex, until it is down-regulated by the anticoagulant pathways. Fibrin molecules make a tangled net of fibers by adhering end-to-end and side-to-side, which immobilizes the fluid portion of blood (causing it to solidify) and also traps the red blood cells.

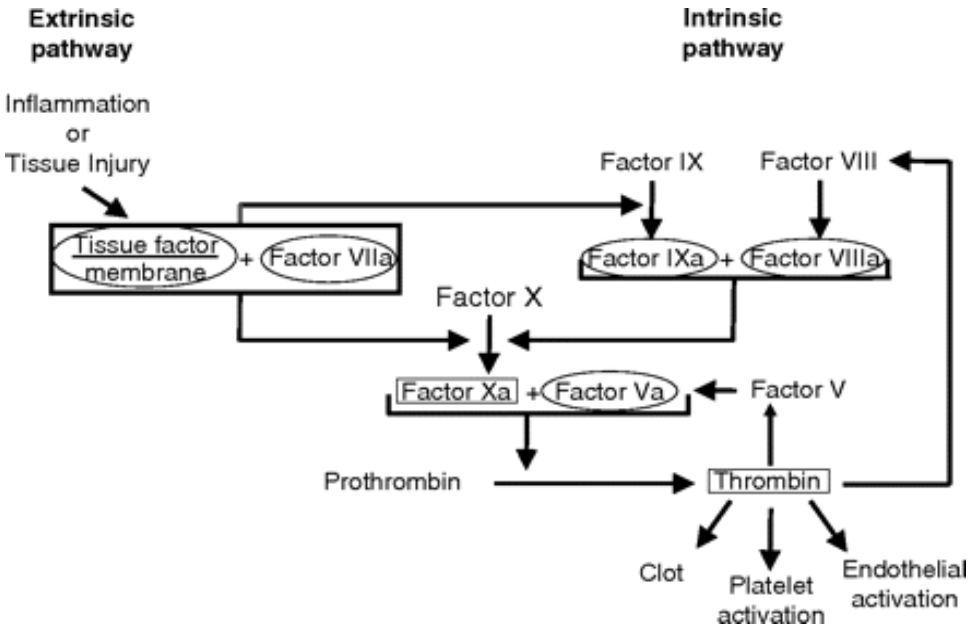


Fig-3: Coagulation cascade (Turpie, 2007).

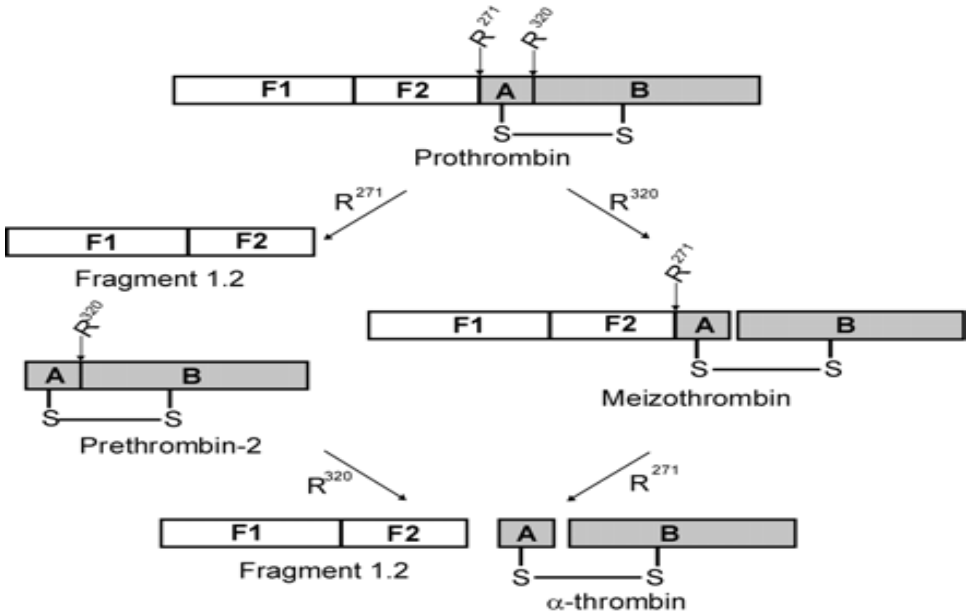


Fig-4: Activation of Prothrombin (Wood *et al.*, 2011)

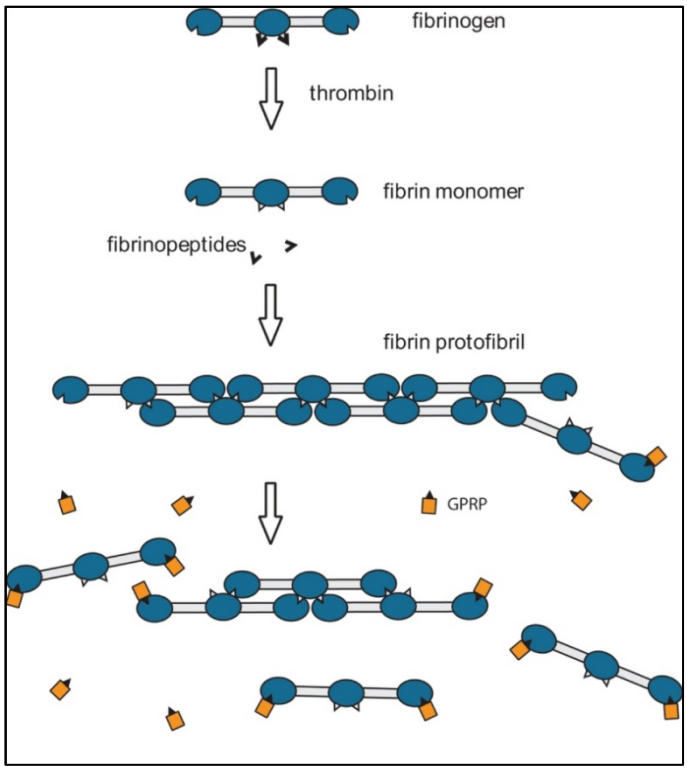


Fig.-5: Activation of Fibrinogen (Chernysh *et al*., 2012)

1.1.4 Dissolution of Fibrin Clots

Degradation of fibrin clots (Fibrinolysis, prevent unwanted clot) is the function of plasmin, a serine protease that circulates as the inactive proenzyme, plasminogen. Plasminogen binds to both fibrinogen and fibrin, thereby being incorporated into a clot as it is formed. Then tissue plasminogen activator (tPA) and urokinase convert plasminogen to plasmin, which then digests the fibrin; the result is soluble degradation product to which neither plasmin nor plasminogen can bind. Following the release of plasminogen and plasmin they are rapidly inactivated by their respective inhibitors.

1.2 Anticoagulants

An anticoagulant is a substance that prevents coagulation of blood. A group of pharmaceuticals called anticoagulants can be used *in vivo* as a medication for thrombotic disorders. Anticoagulants reduce blood clotting. This prevents deep vein thrombosis, pulmonary embolism, myocardial infarction and stroke. Almost

all the haematophagus (blood sucking) insects contains anticoagulants which means they have substances which interferes blood clotting in any step of the coagulant cascade. Therefore, uses of anticoagulant in certain medical condition are vital.

1.2.1 Types of anticoagulants:

There are several different types of anticoagulants found from different sources which are described below:

1.2.1.1 Heparin and derivative substances- Heparin is frequently used in order to prevent life-threatening clotting in many patients, especially those which have had stent implants on their heart. Unlike some other coagulants, Heparin is a biological substance. Although there are many forms of production, it is usually made from pig intestines. The mechanism of function is rather simple; it activates antithrombin III, a substance in our body that blocks thrombin from clotting blood.

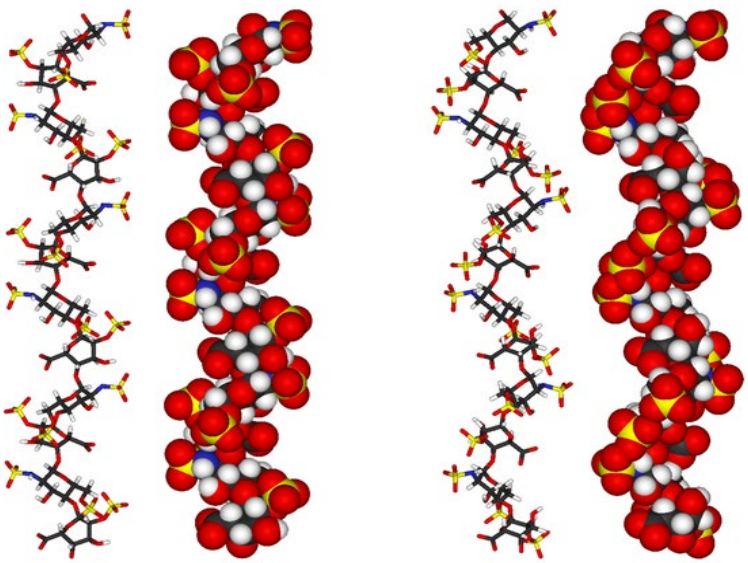


Fig.-6: 3D structure of Heparin (Ferro *et al*., 1990)

Heparin can be administrated by injection or *in vitro* to prevent blood or plasma clotting in or on medical devices. There are several forms of Heparin and the most commonly used is called low molecular weight heparin.

1.2.1.2 Vitamin K antagonists (VKA)- Also called the common oral anticoagulants; these represent a form of anticoagulants which act by blocking the effects of vitamin K (which promotes coagulation). They take 48-72 hours to develop to full effect that is why they should not be used when immediate effect is required. In these cases, it is the best to use heparin. Most common indications for this type of anticoagulants are deep-vein thrombosis (DVT), pulmonary embolism, atrial fibrillation, and mechanical prosthetic heart valves such as stents and different dilatators. The most common complication is of course unstoppable bleeding, especially in patients aged 80 or more. Different types of VKAs are coumarins (4-hydroxycoumarins), warfarin (Coumadin), coumatetralyl, phenprocoumon, acenocoumarol, dicoumarol, tioclomarol and brodifacoum. Among them coumarins are most familiar.

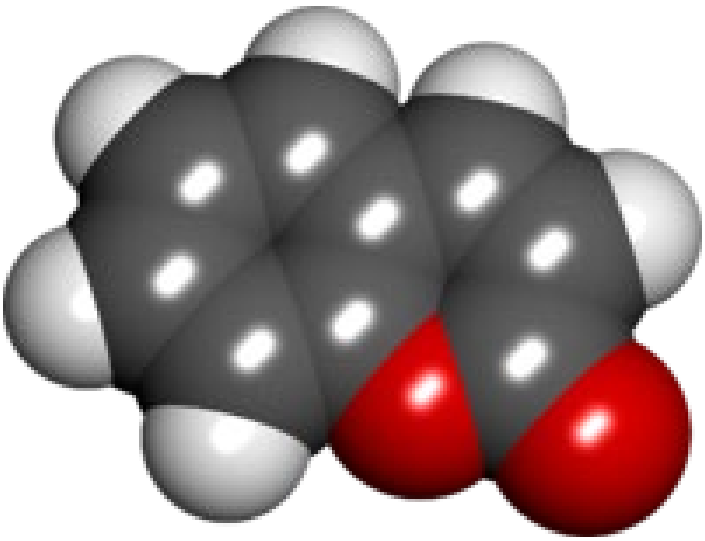


Fig.-7: 3D Structure of Coumarin

1.2.1.3 Low Molecular Weight Heparins (LMWHs)-

LMWHs were developed to overcome some of the drawbacks of unfractionated heparin (Hirsh *et al*., 2001). However, LMWHs must still be administered by injection. It can also be accumulated in patients with kidney impairment. Different types of LMWHs are listed in table-1.

Table- 1 Molecular weight (MW) data and anticoagulant activities of currently available LMWH products (Gray *et al*., 2008):

LMWH	Average molecular weight	Ratio anti-Xa/anti-IIa activity
Bemiparin	3600	8.0
Nadroparin	4300	3.3
Reviparin	4400	4.2
Enoxaparin	4500	3.9
Parnaparin	5000	2.3
Certoparin	5400	2.4
Dalteparin	5000	2.5
Tinzaparin	6500	1.6

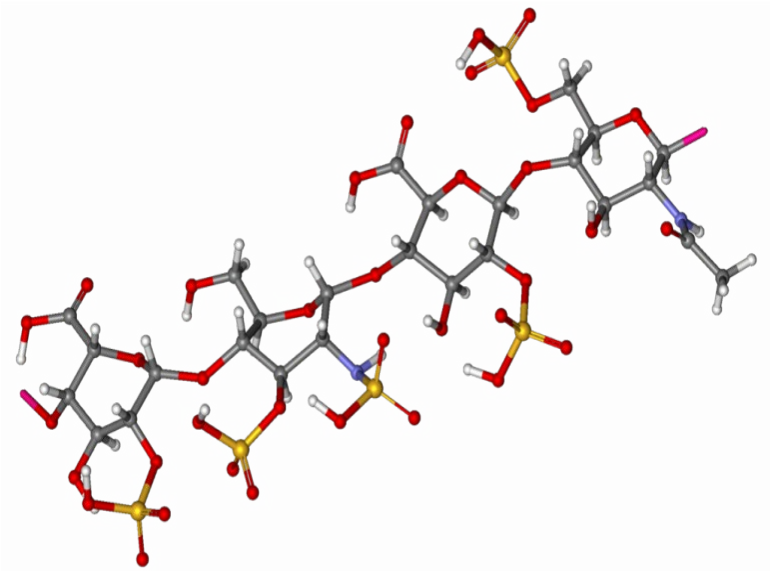


Fig.-8: 3D Structure of Enoxaparin

1.2.1.4 Oral Coagulants- The most important oral anticoagulants are: Warfarin (Coumadin®), Acenocoumarol, Phenprocoumon, and Phenindione. Among them warfarin is more familiar than others.

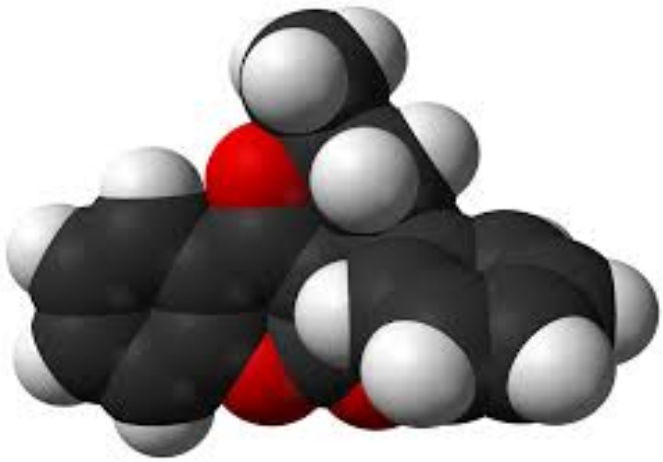


Fig.-9: 3D structure of Warfarin

1.2.1.5 Direct thrombin inhibitors- Some of the most commonly used medications from this group are Argatroban, Lepirudin, and Bivalirudin. There was a minor affair with one medication from this group - an oral direct thrombin inhibitor called Ximelagatran (Exanta®). It has been proven that it can cause severe liver damage and heart attacks. It was denied approval by the Food and Drug Administration, USA in 2006.

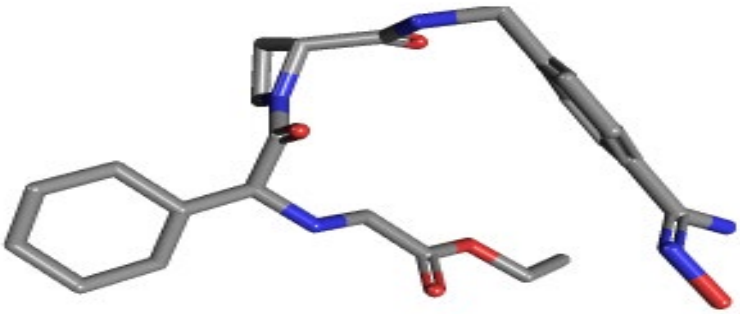


Fig.-10: 3D structure of Ximelagatran

1.3 Hematophagous tick

Ticks are the good source of anticoagulant protein. They locally had known as '*atel'* belongs to two families namely Argasidae and Ixodidae of class Arachnida that are ectoparasite in nature. Ticks are more common in tropical countries and are most abundant on mammals. However, some parasitize birds and reptiles, and occasionally some feed on amphibians. The cattle tick is an external parasite, and is regarded as a significant economic pest of the most cattle industry. Cattle are the main hosts for cattle ticks although they may be found on horses, goats, sheep, deer, camelids and buffaloes.

The cattle tick is widely distributed in Central and South America, parts of the southern USA, Africa, Asia, and northern Australia. The distribution of the cattle tick is largely determined by climatic factors. Cattle tick requires high humidity and ambient temperatures of at least 15-20˚ C for egg laying and hatching. More than a dozen tick species have so far been recorded from Bangladesh infesting cattle, goat, sheep, dog, wild mammals, birds, and lizards (Rahman *et al*., 1985). *Haemaphysalis bispinosa* (flagging, cattle, goats, and dogs), *Rhipicephalus microplus* (cattle, goats), *Rhipicephalus annulatus* (cattle) and *Rhipicephalus sanguineus* (dogs, cattle goats, and flagging) are frequently found in Bangladesh (Fuehrer *et al*., 2012). *R. microplus* and *R. annulatus*are are more common ticks of cattle, whereas *R. sanguineus* is a common tick of dog occasionally attacking cattle and man. However, in many occasions 'tick toxicosis' which is distinct from "tick paralysis" and is characterized by sweating, profuse moist eczema, and hyperanemia of the mucous memberane, has been reported from man and cattle.

1.3.1 Taxonomy

About 867 tick species are known to found throughout the world. They are placed into three families, one of which is Nuttalliellidae, comprise a single species named *Nuttalliella namaqua*. The remaining two families contain the hard ticks (Ixodidae) and the soft ticks (Argasidae). Approximately 80 % of the world's tick fauna are hard ticks (683 species) and the remainders are soft ticks (183 species). The world's soft tick fauna is divided into four genera, namely *Argas, Carios, Ornithodorosand* and *Otobius,* whereas the world's hard tick

fauna consists of 241 species in the genus Ixodes and 442 species in the remaining genera (Camicas *et al*., 1998 and Horak *et al*., 2002).

Nuttalliell anamaqua is found in southern Africa from Tanzania to Namibia and South Africa, which is placed in its own family, Nuttalliellidae. It can be distinguished from ixodid ticks and argasid ticks by a combination of characters including the position of the stigmata, lack of setae, strongly corrugated integument, and form of the fenestrated plates.

Ixodidae are distinguished from the Argasidae by the presence of a scutum or hard shield. Ixodidae nymphs and adults both have a prominent capitulum (head) which projects forwards from the body; in the Argasidae, conversely, the capitulum is concealed beneath the body.

Argasidae family contains 193 species, although currently accepted genera are *Antricola*, *Argas*, *Nothaspis*, *Ornithodoros*, and *Otobius* (Horak *et al*., 2002).

Ixodidae are hard ticks contain 702 species in 14 genera (Horak *et al*., 2002), some are of considerable economic importance as vectors of diseases caused by bacteria such as *Rickettsia* and *Borrelia*. 14 genera of hard ticks are listed in table-2.

Table-2: List of hard tick (Horak *et al*., 2002):

Amblyomma – 130 species	*Haemaphysalis* – 166 species
Anomalohimalaya – 3 species	*Hyalomma* – 27 species
Bothriocroton – 7species	*Ixodes* – 246 species
Cosmiomma – 1species	*Margaropus* – 3 species
Cornupalpatum – 1 species	*Nosomma* – 2 species
Compluriscutula – 1 species	*Rhipicentor* – 2 species
Dermacentor – 34 species	*Rhipicephalus* – 82 species

In hard tick family one of the most common genera, found throughout the world is *Rhipicephalus* that contains 82 species. Among the 82 species there are 12 species are common in Bangladesh (Ghosh *et al*., 2007) and *Rhipicephalus microplus* (Canestrini, 1888) and *Rhipicephalus annulatus* (Say, 1821) are the most abundant among the 12 species.

Scientific classification

Kingdom: Animalia

Phylum: Arthropoda

Class: Arachnida

Order: Ixodida

Family: Ixodidae

Genus: *Rhipicephalus*

Subgenus: *Boophilus*

Species: *R. microplus*

R. annulatus

Binomial name-*Rhipicephalus microplus* and *Rhipicephalus annulatus*

1.3.2 Habitat

Tick species are widely distributed around the world. However, they tend to flourish more in countries with warm, humid climates, because they require a certain amount of moisture in the air in order to undergo metamorphosis, and because low temperatures inhibit their development from egg to larva. Ticks of domestic animals are especially common and varied in tropical countries, where they cause considerable harm to livestock by transmission of many species of pathogens and also causing direct parasitic damage.

For an ecosystem to support ticks, it must satisfy two requirements: there must be a high enough population density of host species in the area, and there must be high enough humidity for ticks to remain hydrated. Due to their role in transmitting Lyme disease, ixodid ticks, have been studied using geographic information systems (GIS), in order to develop predictive models for ideal tick habitats. According to these studies, it was determined that certain features of a given microclimate - such as sandy soil, hardwood trees, rivers, and the presence of cattle - are good predictors of dense tick populations.

1.3.3 Life Cycle

The common life cycle of cattle tick (Fig.-11, 12) is completed in four distinct stages that are described below-

1.3.3.1 The Egg-Mating of hard ticks usually occurs while they are on the host animal. Afterwards the female drops to the ground and, after a brief pre-oviposition period of 3 to 10 days, begin to deposit eggs on or near the earth. The female hard tick feeds once, lays one large batch of eggs sometimes numbering in the thousands, and dies. Most of the soft ticks engorge with blood several times and deposit about 20 to 50 eggs in a batch after each blood meal. Eggs hatch in two weeks to several months, depending upon temperature, humidity and other environmental factors.

1.3.3.2 The Larva-The larvae, or "seed ticks," have only six legs, and the sexes are indistinguishable. Their chances of attaching to a host are precarious, sometimes resulting in prolonged fasts. Despite tolerance to starvation, a very high percentage dies. Some individuals climb on vegetation, waiting for a small rodent to pass within reach. Some actively seek a vertebrate host, being guided by the scent of the animal. After a blood meal, the engorged larvae usually drop to the soil and molt to the eight-legged nymph stage. The larvae of one-host ticks remain on the host to molt.

1.3.3.3 The Nymph-The nymph has eight legs like the adult but has no genital opening. This stage also must undergo a critical waiting period for a suitable host. After engorgement, the nymph drops from the host, molts, and becomes an adult. Nymphs may rest for long periods before becoming adults. Some species of hard ticks live less than one year while others live three years or more. Each time a tick leaves its host it risks its survival on finding another host. Some species have the advantage of molting on the host. For example, the cattle tick is a one-host tick. Multiple-host tick species are able to exist because of their great reproductive capacity and their ability to survive for a long time without food. Hard ticks have only one nymphal instar, the nymph becoming an adult after molting. Soft ticks may have several nymphal instars.

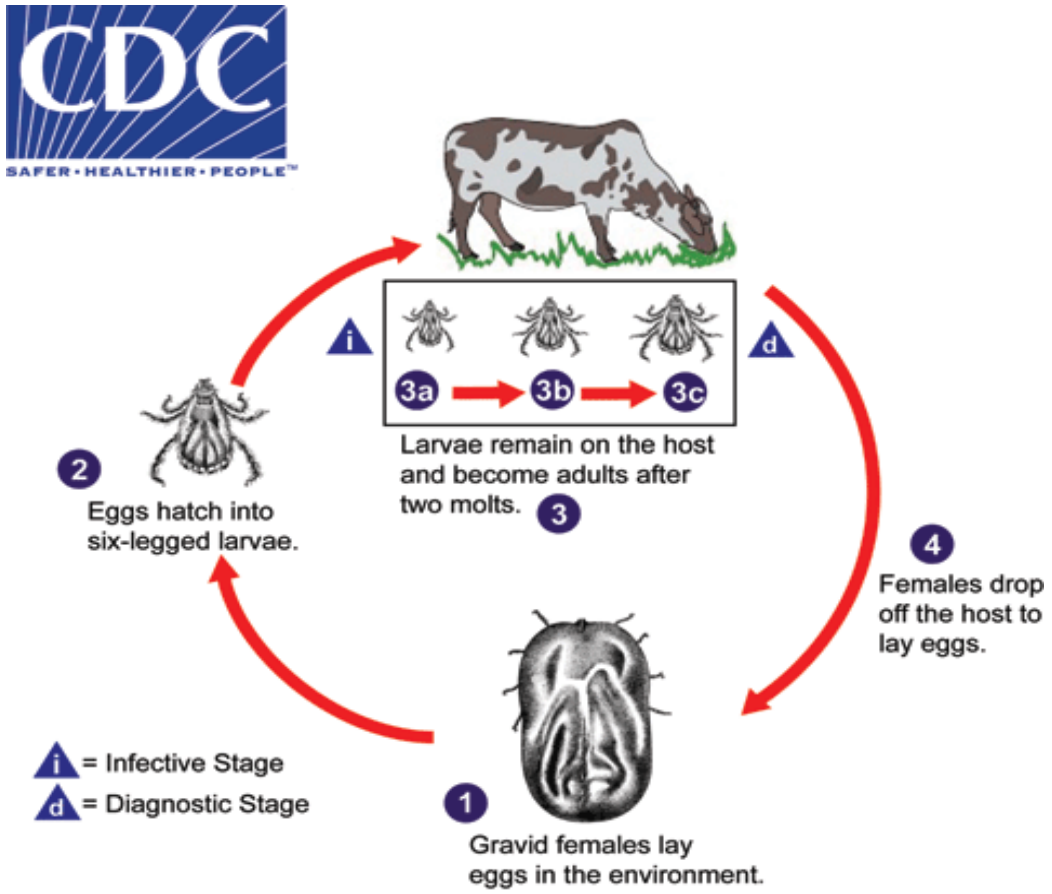


Fig.- 11: Life cycle of cattle tick *Rhipicephalus microplus* (one host) Source: Centers for Disease Control and Prevention, http://www.dpd.cdc.gov/dpdx

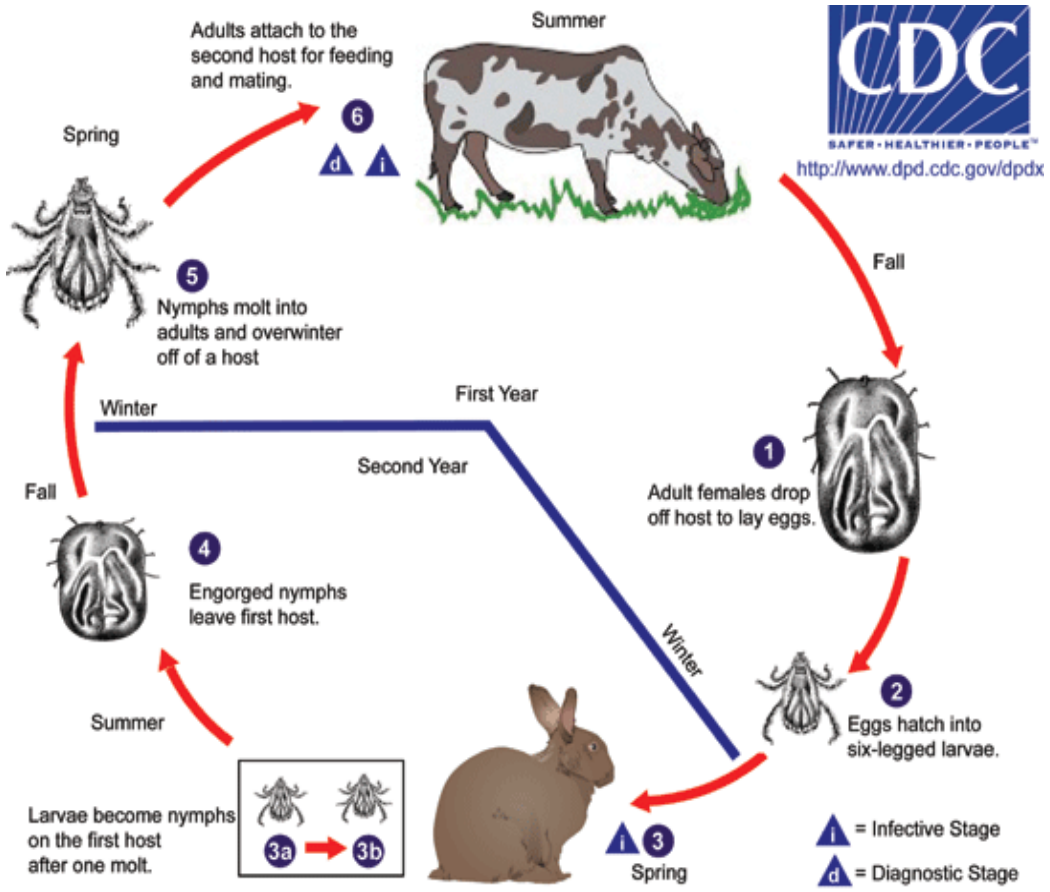


Fig.- 12: Life cycle of cattle tick *R. annulatus* (three host) Source: Centers for Disease Control and Prevention, http://www.dpd.cdc.gov/dpdx

1.3.3.4 The Adult-Typically, the nymph molts after engorgement and becomes an adult. Sex then is distinguishable for the first time as the female hard tick differs from the male in having a small scutum. The sex of soft ticks may be determined by the shape of the genital opening located between the second pair of legs. In male soft ticks the genital opening is almost circular, while it is oval and definitely broader than long in female specimens. Unlike mosquitoes, both male and female hard ticks are blood suckers, and both require several days feeding before copulation. After the male hard tick becomes engorged, he usually copulates with one or more females and then dies. Following copulation, the female tick drops to the ground. The eggs require several days to develop. Then she begins oviposition. After a few more days, her life's mission accomplished, the spent female hard tick also dies. The female soft tick may lay several small batches of eggs but she requires another blood meal before each episode of oviposition.

1.3.4 Impact of cattle tick in cattle breed

Bangladesh is basically an agrarian country with 84% of the total population living in rural areas and 55.6% directly and indirectly engaged with agricultural activities. Livestocks play an important role in the agricultural economy. About 24.6% GDP comes from agricultural sector and livestock contributes 3.5% (FAO, 2004). Unfortunately there are no accurate and recent estimates available of the damages caused by disease vectors. Only one estimate of the Bangladesh Agricultural University Veterinary hospital puts the losses at 15.2% of the cattle populations due to mosquito, fly and ticks (Rahman *et al*., 1972). The rich bio-diversity and varied climates puts Bangladesh at particular risk for disease epidemics. No particular emphasis has been given to research on ticks and tick born diseases. However the prevalence of tick-borne pathogens, the distribution and the incidence of ticks and tick transmitted diseases are unknown. There is a clear need to locate the tick endemic areas and to identify the severity of tick problem. Ticks and tick-borne pathogens are known to exist in Bangladesh. In the Indian subcontinent, several species of tick-borne pathogens in livestock have been described. Classical diagnostic methods, like

blood smears and salivary gland smear of ticks, are used to determine the presence of haemoparasites and very limited also used for research purpose in Bangladesh. Due to morphological similarities it is sometimes difficult to distinguish between different species. Molecular diagnostic methods, so far not yet used in Bangladesh, could help to determine the prevalence and incidence of tick-borne diseases in cattle in Bangladesh. PCR and RLB-PCR are new molecular techniques now widely used for the detection of tick-borne diseases from blood and ticks in cattle. The training and collaboration in a mutual partnership should help to use these methods for the detection of haemoparasites in the bovine blood and in the haemolymph of ticks of cattle in Bangladesh.

Tick paralysis is a disease of man and animals characterized by an acute ascending flaccid motor paralysis caused by the injection of a toxin by certain ticks.

Adult female ticks, and often nymphs, while engorging inject toxins, usually at the later stages of feeding blood. In particular, ticks which attach to the head and neck of the host usually inject toxins. The toxin production is concomitant with egg production in females. Toxin has also been detected in suspension of crushed eggs which when injected in animals also produce paralysis. The degree of paralysis is proportionate to the length of time the tick has been feeding. Frequently, a number of ticks attach themselves to host. Removal of the ticks is usually followed by recovery, provided the cardiac and respiratory centres have not been affected. Tick paralysis is generally observed in isolated cases of individual animals/man but it can also affect herds of animals and can cause severe losses if untreated.

Tick paralysis affecting cattle, sheep, goat, dog, pig, wild ruminants, and guineapig has been widely documented in Africa, Asia, Europe and Australia. Tick species which are increminated to tick paralysis are Argaspersicus and Ornithodoroslahorensis among the soft ticks (Argasidae) and several species of Ixodes, Demacentor, Rhipicephalus, Haemaphysalis, Hyalomma, and Amblyomma among the hard ticks (Ixodids).

1.4 Aim of the present study

1. Isolation and collection of salivary glands
2. Extraction of total protein from salivary glands
3. SDS PAGE profiling of the crude salivary gland extract
4. Evaluation of anticoagulant activity of the crude salivary gland extract and assessing the potentiality of the anticoagulant protein to develop as a commercially viable drug source
5. RP-HPLC purification of the crude salivary gland extract in search of anticoagulant proteins
6. Evaluating the potency of the HPLC peaks for anticoagulant activity

CHAPTER 2

LITERATURE REVIEW

Anticoagulants are such types of protein that delay blood clotting. Medications have been used for more than 70 years to prevent and treat these deadly blood clots. Widely used traditional therapies are associated with significant drawbacks. However, for centuries, research work had been carried out to find out novel anticoagulant drug to solve the problem of thrombotic disorders.

In 1916, heparin, an anticoagulant was accidentally discovered by Jay McLean, a second-year medical student at Johns Hopkins University, was working under the guidance of the eminent scientist William Howell investigating procoagulant preparations, when he isolated a fat soluble anticoagulant in canine liver tissue. The discovery of heparin was not followed by immediate practical use. Twenty years later in 1938, its clinical utility in the treatment of post traumatic venous thrombosis of the lower extremities was recognized, by the Canadians Charles Best and Gordon Murray (Murray *at al*., 1938). Heparin is still used for the treatment of venous thromboembolism (Hirsh *et al*., 2001) and is effective if used properly. However, heparins require administration by injection or infusion, which can be inconvenient and cause discomfort. Besides, some patients experience an adverse reaction known as HIT (heparin-induced thrombocytopenia) by using heparin (Jang *et al*., 2005). Therefore, novel anticoagulant is badly needed until its discovery.

Later on, Karl Paul Link first time discovered vitamin K antagonists (VKA) in 1933 (Griminger, 1987) from sweet clover a legume weed. Since its unexpected discovery in 1933, warfarin, acenocoumarol (the first oral anticoagulants) and, more generally, vitamin K antagonists (VKAs) have been widely used in clinical practice. VKAs were first used as rodenticides. There was a growing use of VKAs in humans in 1955 after President Eisenhower received them after a heart attack. However, the use of VKA is still challenging because they are characterized by a narrow therapeutic index and a great inter-individual variability in the dose response to the drug. Another cause is that, they have many food and drug interactions.

In the year 1997, Weitz developed an important class of antithrombotic compounds, low molecular weight heparins (LMWHs) to overcome some of the drawbacks of unfractionated heparin (Weitz, 1997). However, LMWHs must still be administered by injection and can also accumulate in patients with kidney impairment. So there is a crucial need to develop another type of anticoagulant to overcome the limitations of traditional anticoagulants to prevent and or treat venous and arterial thromboembolic conditions.

In 2005, Di Nisio developed another anticoagulant that directly inhibits thrombin, the enzyme that promotes clot formation (Di Nisio *et al*. 2005). Ximelagatran and Dabigatran are two novel anticoagulants of this type. Ximelagatran approved in Europe in 2004 and it was withdrawn in 2006 due to severe liver damage in some patients. Dabigatran approved in Europe in 2008 for the prevention of venous and arterial thromboembolic (VTE) in patients undergoing hip or knee replacement. Still there are some problems to use this type of anticoagulants and require frequent monitoring and subsequent dose adjustment. Therefore, there is an imperative need for novel, oral agents with a predictable anticoagulant action.

In 2007, Turpie developed an oral anticoagulant, Xarelto (rivaroxaban), the first direct F-Xa inhibitor to be approved for the prevention of VTE in adult patients undergoing elective hip or knee replacement surgery (Turpie, 2007). Since then, ‘Xarelto’ has been approved to protect patients across more VAT diseases than other novel oral anticoagulants.

Another type of oral anticoagulant ‘Apixaban’ approved recently in 2011, which is used now a day for its wide range of application (Wong *et al*. 2011). It is hoped that, before long, these anticoagulants will allow us to enter an era of convenient, oral anticoagulation, without the need for regular monitoring or dose adjustment.

However, productions of these above anticoagulants are currently expensive and they are still fairly narrow in their effectiveness. Besides, these anticoagulant drugs have long polypeptide chain, which cannot excrete through the kidney nephron causing kidney failure. For this reason there is still some

chance to evaluate potential new anticoagulant proteins to develop as a commercially viable drug source.

From the beginning of the civil human society, scientist works hard to find out promising anticoagulant drug from various plant and animal sources to solve thrombotic problems.

In 1938, Chargaff works on the chemistry of blood coagulation factor VII protamines, which is used as an inhibitor of blood coagulation (Chargaff, 1938).

Seven years later, Chargaff isolated a thromboplastic protein from human organs in 1945 (Chargaff, 1945).

In the year 1987, Ribeiro, describe the role of saliva in blood-feeding by arthropods (Ribeiro, 1987).

Very next year Gauci *et al*. isolated and characterised an allergic substance named allergens from the salivary glands of an Australian paralysis tick *Ixodes holocyclus* (Gauci *et al*., 1988).

Atoda and Morita isolated a novel blood coagulation factor FIX having anticoagulant activity from snake venom in 1989 (Atoda *et al*., 1989).

Later on, Jaworski and his co-worker identified salivary gland antigens from unfed and early feeding females of a lone star tick *Amblyomma americanum* and compared it to *Ixodes dammini* and *Dermacentor variabilis* salivary glands antigen in 1990 (Jaworski *et al*., 1990).

In the same year, Jacobs *et al*. isolated and characterised of a coagulation factor Xa inhibitor from a black fly (*Simulium vittatum*) salivary glands. According to them, the gland extract inhibited both thrombin and factor Xa (Jacobs *et al*., 1990).

Limo and his co-worker isolated, purified and characterised an anticoagulant protein of 65 kDa from the salivary glands of the ixodid tick *Rhipicephalus appendiculatus* in 1991(Limo *et al*., 1991). They purified it by gel filtration on Sephadex G-100, ion exchange on DEAE-cellulose, aprotinin-Sepharose, and by high-pressure-liquid size-exclusion chromatography.

In 1994, Abebe and his coworkers showed the anticoagulant activity of the salivary gland extracts of four female black flies species i.e., *Simulium vittatum* Zetterstedt, *Simulium argus* Williston, *Simulium metallicum* Bellardi, and *Simulium ochraceum* Walker (Abebe *et al*., 1994).

Next year, Ribeiro *et al*. purified and characterised a novel salivary anticoagulant named prolixin-S (nitrophorin-2) from the salivary glands of the blood-sucking bug *Rhodnius prolixus* (Ribeiro *et al*., 1995).

A factor Xa-directed anticoagulant from the salivary glands of the yellow fever mosquito *Aedes aegypti* was discovered by Stark and James in the year 1995 (Stark *et al*., 1995).

In the year of 1996, the scientist (Sun J *et al*., 1996) of Mie University School of Medicine, Tsu, Japan extracts an anticoagulant protein, Prolixin-S from *Rhodnius prolixus* which is a specific inhibitor of intrinsic blood coagulation pathway.

In the same year scientist from Institute of Radiation Medicine, Academy of Military Medical Sciences, isolated new steroidal saponins (Peng JP *et al*., 1996) from *Allium sativum* which inhibits platelet aggregation, fibrinolysis and blood coagulation.

At the same time, Wang and his co-workers isolated ‘Variabilin’, a novel RGD-containing antagonist of glycoprotein IIb-IIIa and platelet aggregation inhibitor from the hard tick *Dermacentor variabilis* (Wang *et al*., 1996).

In 1997, Zhu *et al*., worked on isolation and characterization of ‘americanin’, a specific inhibitor of thrombin, from the salivary gland extracts of the lone star tick *Amblyomma americanum* L. (Zhu *et al*., 1997).

In the same year, (Fuentes-Prior *et al*., 1997) from Max-Planck-Institut für Biochemie, Abteilung Strukturforschung, D-82152 Martinsried, Germany, isolated a potent and specific thrombin inhibitor Triabin, (142 residues) from the blood sucking bug *Triatoma pallidipennis*.

In the same year, a group of scientist (Cappello *et al*., 1998) worked on tsetse thrombin inhibitor from the salivary glands and gut tissue of *Glossina morsitans morsitans*, which showed blood meal induced expression.

The scientist of Arizona University, Department of Biochemistry, USA, isolated a heme-based proteins nitrophins, (Weichsel *et al*., 1998) a salivary gland protein from the blood sucking insect *Rhodnius prolixus* inhibits platelet aggregation.

During the end of the year of 1998, Waidhet-Kauadio and his coworks purified and characterized of a thrombin inhibitor from the salivary glands of a malarial vector mosquito, *Anopheles stephensi* (Waidhet-Kauadio *et al*., 1998).

In the year of 1999, Tikki along with a group of scientists worked on female tick *Hyalomma marginatum marginatum* salivary glands and preliminary studied on the protein changes during their feeding process and antigens recognized by repeatedly infested cattle (Tikki *et al*., 1999).

In the same year Mende *et al*., from the Research Unit Pharmacological Hemostaseology, Medical Faculty, Friedrich Schiller University Jena, Germany, isolated Dipetalogastin, a cDNA coding for the thrombin inhibitor from the stomach of the blood sucking insect *Dipetalogaster maximus* (Mende *et al*., 1999).

One year later Mulenga and his coworker described the critical issues in tick vaccine development and identified and characterised potential candidate vaccine antigens (Mulenga *et al*., 2000).

Kazimírová *et al*., later on worked on the identification of anticoagulant activities in salivary gland extracts of four horsefly species (Diptera, tabanidae) in 2001(Kazimírová *et al*., 2001).

In 2002, Basanova and his coworkers discovered some regulators from some bloodsucking animals that are used in vascular-platelet and plasma hemostasis(Basanova *et al*., 2002).

In the same year, Zavalova *et al*. developed some fibrinogen-fibrin system regulators from some bloodsuckers (Zavalova *et al*., 2002).

Markwardt in 2002 described the historical perspective of the development of thrombin inhibitors (Markwardt, 2002).

Kazimirova in 2002 examined the anticoagulant activities in salivary gland extracts of 19 species of tabanid flies (Kazimirova, 2002). He used standard

coagulation screen assays to determine thrombin time, prothrombin time and activated partial thromboplastin time. He found that SGE of most species considerably prolonged human plasma clotting time in a dose-dependent manner.

In 2005, Yajnavalka Banerjee and his coworkers isolated and characterized a snake venom anticoagulant protein complex named 'Hemextin AB Complex' from *Hemachatus haemachatus* (Banerjee *et al*., 2005).

One year later, Johne described that, platelets promote coagulation factor XII-mediated proteolytic cascade systems in plasma in 2006 (Johne *et al*., 2006).

In 2006 Sasaki *et al.* of Escola Paulista de Medicina, Brazil,describe the Kunitz type serine proteinase inhibitors from the mid gut of *Rhipicephalus microplus* (Sasaki *et al.*, 2006).

In the year of 2007, the scientists of Pukyong National University isolate a novel anticoagulant protein from *Tegillarca granosa* (Won-Kyo Jung *et al*., 2007).

In the year of 2007 the scientist (Ricci *et al.*, 2007) from Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul, Brazil. First time isolated a thrombin inhibitor from the gut of *Rhipicephalus microplus*.

Recently, scientists for National University of Singapore have identified an anticoagulant peptide from tropical bont tick (Cho Yeow Koh *et al.*, 2007). The species they worked on were collected from Slovenia. According to their finding, tick saliva contains potent antihemostatic molecules that help ticks obtain their enormous blood meal during prolonged feeding. They isolated thrombin inhibitors present in the salivary gland extract from partially fed female *Amblyomma variegatum*, the tropical bont tick, and characterized the most potent, variegin, one of the smallest (32 residues) thrombin inhibitors found in nature.

The researchers (Liao *et al.,* 2009,) from Kagoshima University, Korimoto, Kagoshima, Japan. Isolated a thrombin inhibitor Hemalin (20 Kda) from the midgut of *Rhipicephalus microplus*.

In the year 2011, Asif *et al*., worked on tick and described the immune response and tick rejection pattern of midgut and salivary gland vaccines against locally prevalent *Boophilus microplus* ticks (Asif *et al*., 2011).

Recently researchers (Soares *et al*., 2012) from Departamento de Bioquímica, Escola Paulista de Medicina, Universidade Federal de São Paulo, Rua 3 de Maio, São Paulo, SP, Brazil, isolated a thrombin inhibitor Boophilin from *Rhipicephalus micoplus* midgut in 2012.

Borah *et al*. determined the anticoagulation activity of salivary gland extract of oriental black fly *Simulium indicum* in 2014 (Borah *et al*., 2014).

However, there is no report on haematophagus insect from our region though we have a number of species of them. So it will be an outstanding research work to found out novel anticoagulant peptides as a potential drug source from the blood sucking insects of our country.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The following materials were used for the purpose of my present study-

3.1.1 Experimental Materials

Cattle Tick *Rhipicephalus microplus* and *Rhipicephalus annulatus* were used as experimental materials. Near about 1000 female insects of *Rhipicephalus microplus* and *Rhipicephalus annulatus* (Fig.-13) were collected from different cattle farms of Rajshahi, Natore, Pabna and Chapainawabganj.

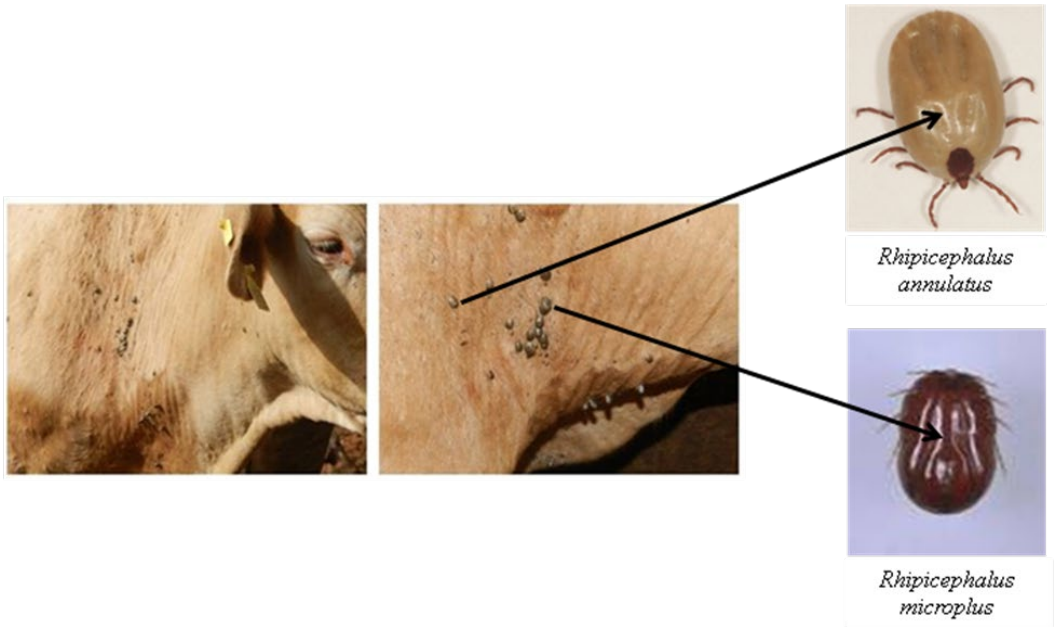


Fig.-13: Cattle ticks (*Rhipicephalus microplus* and *Rhipicephalus annulatus*)

3.1.2 Equipment

- Centrifuge machine
- Conical flask
- Electric balance

- Eppendorf tube
- Fine forceps
- Needle
- Oven
- Petri dish
- pH meter
- Pipette and Micropipette
- Refrigerator
- Scalpel
- Stereo microscope etc.

3.1.3 Chemicals

3.1.2.1 Phosphate Buffered Saline (PBS) 1X

- 0.01 M Disodium Phosphate
- 0.0027 M Potassium Chloride
- 0.137 M Sodium Chloride
- 0.0018 M Monopotassium phosphate
- pH 7.4 at 25 °C

3.1.2.2 SDS Sample Buffer 2X

- 3.55 ml de-ionized water
- 1.25 ml 0.5 M Tris-HCl, pH 6.8
- 2.5 ml glycerol
- 2.0 ml 10% (w/v) SDS
- 0.5 ml β-Mercaptoethanol
- 9.5 ml Total Volume
- Store at room temperature

3.1.2.3. 10% (w/v) SDS

- 10 g SDS
- 100 ml deionized water with gentle stirring
- Store at room temperature

3.1.2.4. 150 mM NaCl Solution

- 0.87 g NaCl
- 100 ml deionized water with gentle stirring
- Store at 4° C temperature

3.2 Methods

3.2.1 Collection of Cattle Tick

Cattle ticks which are used in the current project were collected (Fig.-14) from the local cattle farms of four districts of Rajshahi division. Domestic animals such as cows, goats were examined for ticks and were collected. Partially fed ticks were preferred for our experiments. Hand picking method was utilized for collection of the ticks although gloves were used for personal protection. After collection, ticks were kept in plastic vials in normal temperature with the opening of the bottle covered with thin cheese cloth. Sufficient aeration was maintained.

Fig.14: Cattle ticks collected from different cattle farm

3.2.2 Dissection and Isolation of Salivary Glands

A step-by-step procedure for the dissection of the cattle ticks (*Rhipicephalus sp*) and examine the internal morphology to identify salivary glands was followed mentioned by different scientists (Palate-1A, 1B, 1C). Since field-collected ticks are potentially infected with the variety of diseases, so some precautions were necessary. Hand gloves as well as lab coats were put on during experiment. The dissection process was performed under stereomicroscope. The dissection process is described in the following steps-

- Just before the dissection a petri dish was prepared by pouring melted paraffin into the bottom and allowing it to cool and to solidify.
- Then the center of the paraffin coated petri dish was heated briefly (a heated spoon is used to melt the paraffin).
- Once the paraffin is melted, a tick was gently grasped with forceps, placing it into the heated paraffin thereby immobilizing it and restraining its legs.
- Subsequently the tick was covered with a drop of phosphate buffered saline (PBS, Palate-1A). This is an important step because it prevents desiccation of the tissues.
- Then the scutum was removed with a micro scalpel by cutting across the dorsal shield at the anterior point, just distal to the capitulum (Palate-1B).
- Cutting was continued around the edge of the scutum, inserting the micro scalpel into the groove just inside the festoons. Dorsal exoskeleton was lifted by using forceps held in one hand while carefully dissecting the attached muscles and connective tissue with the micro scalpel in the other hand. After removing the scutum complete gut was shown (Palate-1C).
- Digestive system, nervous system and other unnecessary parts of the tick were removed. Salivary gland near the midgut was shown (Palate-1D).
- A clear grape like structures were observed at the proximal end called anterior salivary gland (Palate-1E, 1F). Another set of salivary gland were also observed at midgut.

Plate 1. Dissection of the salivary glands of *Rhipicephalus sp*.

A. Female tick covered with PBS

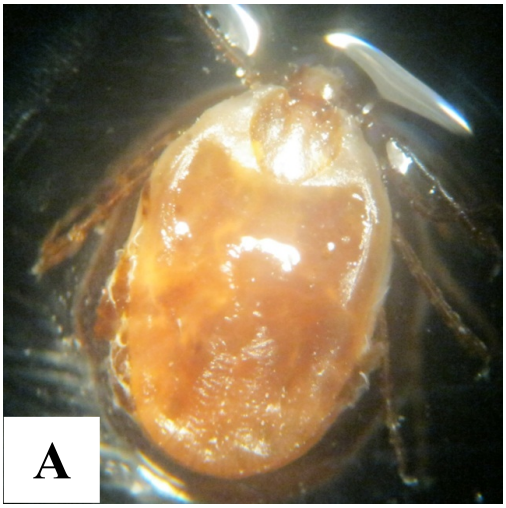
B. Female scutum removed

C. Scutum removed,showing complete gut (Arrow)

D. Salivary gland near the midgut (Arrow)

E. Grape like Salivary glands (Arrow)

F. Salivary glands close up view (Arrow)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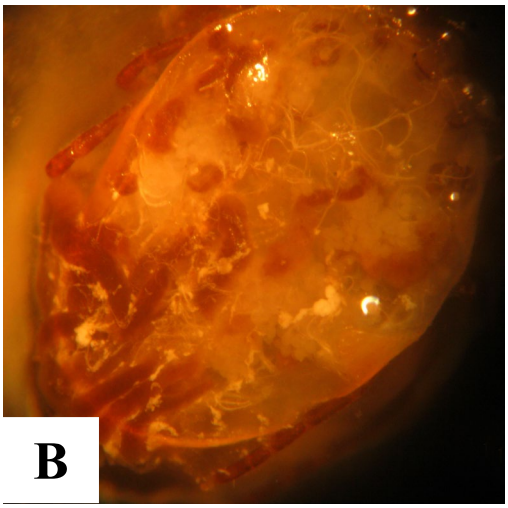
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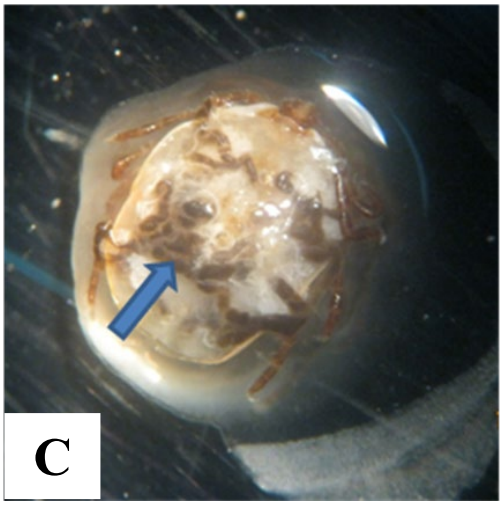
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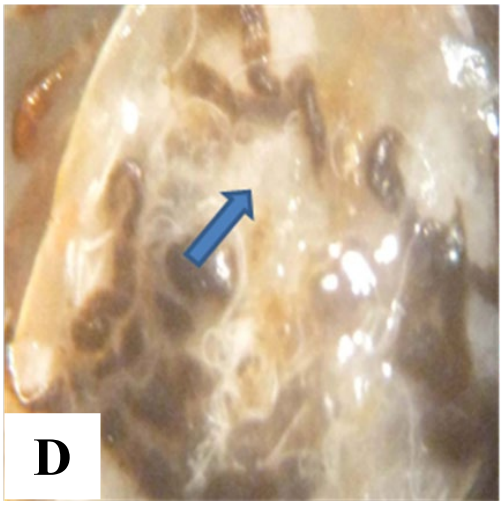
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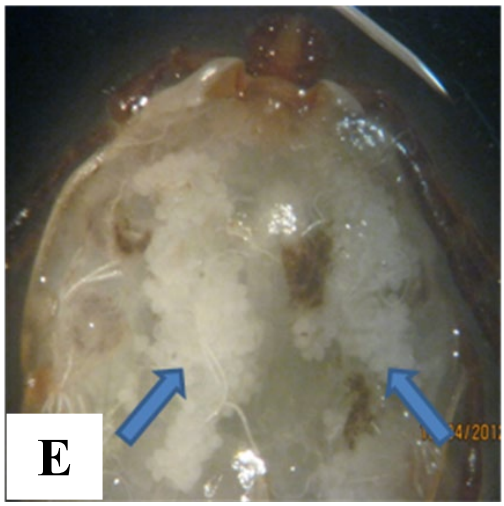
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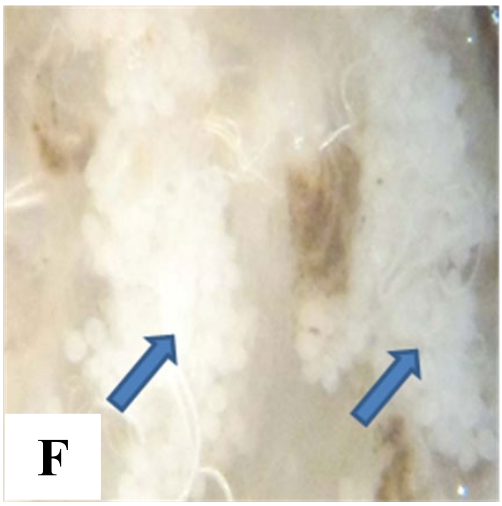
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3.2.3 Storage of Salivary Glands

The salivary glands were picked using a sterile needle and stored it in 1.5 ml eppendorf tube containing phosphate buffered saline. 50 pairs of salivary glands were preserved in 1 ml PBS solution and were stored at -20° C.

3.2.4 Protein Extraction from Salivary Glands

The procedure of the extraction of the crude protein from the salivary gland of the cattle ticks *Rhipicephalus sp.* are discussed below:

3.2.4.1 Reagents

- Liquid Nitrogen
- 150 mM Nacl
- SDS sample buffer

3.2.4.2 Protocol

- Salivary glands which were collected from partially fed female cattle ticks stored at -20º C. Then centrifuged the salivary glands at 10,000 rpm for 30 seconds.
- Pellet contained the salivary glands and the supernatant was thrown away.
- Then liquid nitrogen was added to the centrifuge tube and crashed the salivary glands pellet in liquid nitrogen for homogenization.
- Then 200 µl of 150 mM NaCl solution was added to the above centrifuge tube and was incubated at room temperature for 5 minutes.
- Then span the salivary glands at 10,000 rpm for 10 minutes. There were two layers appeared. Then the supernatant was taken carefully in an autoclaved eppendorf tube using a micropipette without disturbing the pellet. The supernatant contained the desired crude protein. The pellet contained the debris.

- Then resuspended the pellet in 200 µl of 150 mM NaCl solution and incubated it at room temperature for 5 minutes. Then the pellet was properly crashed again.
- Then span the tube at 10,000 rpm for 5 minutes. The supernatant was collected carefully in an eppendorf tube using a micropipette without disturbing the pellet.
- The eppendorf tube containing the crude protein was stored at 4º C for further use.

3.2.5 Estimation of Protein of Salivary Glands

3.2.5.1 Determination of Optical Density (O.D. at 280 nm) vs. Protein Concentration by Folin-Lowary Method (Lowry *et al.*, 1951).

3.2.5.1.1 Reagents

a. Alkaline sodium carbonate solution (20 g/L Na2CO3 in 0.1M NaOH solution).

b. Freshly prepared copper sulphate and sodium potassium tartrate solution (5 g/L CuSO4.5H2O in 10 g/L Na-K tartrate).

c. Alkaline solution: Mixture of solution A and B in the proportion of 50:1 respectively.

d. Folin-Cicolteau's reagent (Diluted with equal volume of H2O, just before use).

e. Standard protein (Bovine serum albumin 10 mg/100 ml in dist. H2O) solution.

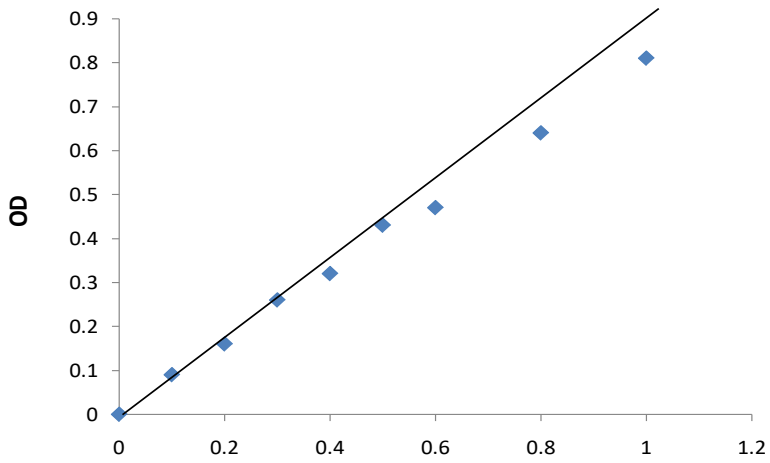
3.2.5.1.2 Method

For the construction of standard curve 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 and 1.0 ml of the standard protein solution were taken in different test tubes and made upto the volume 1 ml by distilled water.

The protein solution (1 ml) was also taken in duplicate in different test tubes and 5 ml of the alkaline solution (solution-c) was added to the standard protein solution in different test tubes and mixed thoroughly. It

was allowed to stand at room temperature for 10 minutes. Then 0.5 ml of diluted Folin-Ciolteau's reagent was added rapidly with immediate mixing and left for 30 minutes. The dark blue color formed was measured at 650 nm against the appropriate blank. By applying the same procedure described above, the absorbance of protein solution was measured and a graph was constructed by plotting concentration against absorbance (O.D) and from the graph the concentration of protein was determined.

3.2.5.2 Standard curve



Concentration

Standard curve of BSA for the determination of protein concentration

3.2.6 SDS-PAGE of SGE

3.2.6.1 Reagents

1. 30% Acrylamide solution:

- 29.2 grams acrylamide
- 0.8 grams bis-acrylamide
- Fill up to 100 ml in distilled water and filter

- Store at 4º C
- The solution is stable for 12 months at 4º C in dark

2. Separating gel buffer: 1.5 M Tris HCl, pH = 8.8

- Tris base 18.15 g/100 ml
- Store at 4º C

3. Stacking gel buffer: 0.5 M Tris HCl, pH = 6.8

- Tris base 6.05 g/100 ml
- Store at 4º C

4. 10% (w/v) SDS

- 10 g SDS
- 100 ml deionized water with gentle stirring
- Store at room temperature

5. 0.5% Bromophenol Blue

- 2.5 mg
- 0.5 ml water

6. 2X Sample Buffer (SDS Reducing Buffer)

- 3.55 ml deionized water
- 1.25 ml 0.5 M Tris-HCl, pH 6.8
- 2.5 ml glycerol
- 2.0 ml 10% (w/v) SDS
- 0.2 ml 0.5% (w/v) bromophenol blue
- 9.5 ml total volume
- 0.500 ml β-Mercaptoethanol
- Store at room temperature

7. 10x Electrode (Running) Buffer, pH 8.3 (makes 1 L)

- 30.3 g Tris base
- 144.0 g Glycine
- 10.0 g SDS
- Dissolve and bring total volume up to 1,000 ml with deionized water
- Do not adjust pH with acid or base
- Store at 4°C. If precipitation occurs, warm to room temperature before use

8. 10% APS (fresh daily)

- 100 mg ammonium persulfate
- Dissolved in 1 ml of deionized water
- Though better to prepare fresh, but still it may be stored at 4°C for several weeks

9. Staining Solution (0.05%)

	1000 ml	200 ml	100 ml
Milli-Q water	500 ml	100 ml	50 ml
Methanol	400 ml	80 ml	40 ml
Glacial Acetic Acid	100 ml	20 ml	10 ml
Coomassie R-250	500 mg	100 mg	50 mg

Bio Rad recommendation stain 1/2 hour with 0.1% coomassie blue R-250 in fixative (40% MeOH, 10% HOAc). But we can use 0.05% and stain for 1 hour.

10. De-staining Solution

	1000 ml	200 ml	100 ml
Milli-Q water	500 ml	100 ml	50 ml
Methanol	400 ml	80 ml	40 ml
Glacial Acetic Acid	100 ml	20 ml	10 ml

Destain with several changes of 40% MeOH + 10% HOAc to remove background (usually 1 to 3 hr).

3.2.6.2 12% SDS resolving/separating and stacking gel

	Resolving/separating	Stacking
Deionized water	3.35	3.35
Tris HCL	2.5 (1.5 M, pH 8.8)	2.5 (0.5 M, pH 6.8)
30% Bis-Acrylamide	4.0 ml	4.0 ml
10% (W/V) SDS Solution	100µl	100 µl
10% APS	50 µl	50 µl
TEMED	5.0 µl	10.0 µl
Total	10 ml	10 ml

3.2.6.3 Protocol

- **Assembling the glass plate**
  - The glass plate was assembled on a clean surface. The longer glass plate was laid down first and then two spacers of equal thickness were placed along the rectangular plate. Then the shorter glass plate was placed on top of the spacers so that the bottom ends of the spacers and glass plates were aligned.

- Then the four screws on the clamp assembly were loosed and stand it up so that the screws were facing away from me. Then the glass plate sandwich was firmly grasped with the longer plate facing away from me and gently slid it into the clamp assembly. Then top two screws were tightened of the clamp assembly.

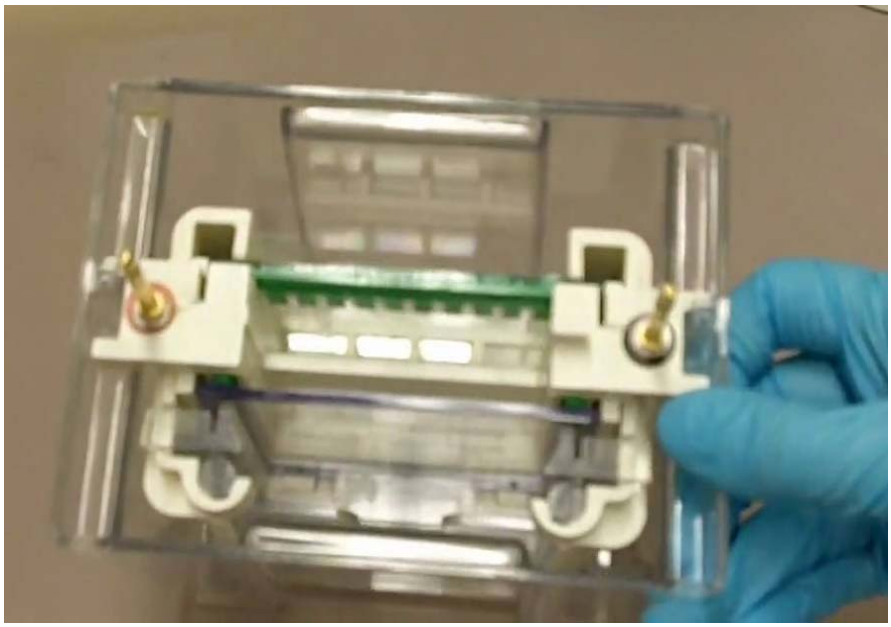


Fig.-15: Protein Gel Electrophoresis Equipment (Polyacrylamide)

- The clamp assembly was then placed into the alignment slot of the casting stand so that the clamp screws faced away from me. The top two screws were loosed to allow the plates and spacers to sit firmly against the casting stand base. Finally, all the screws were tightened gently.
- Then the completed sandwich was pulled from the alignment slot. Then checked it again that the plates and spacers were aligned. If did not, realign the sandwich as in the previous three steps. Before transferring the clamp assembly to the casting slot, the alignment of the spacers was rechecked. This was done by inverting the gel sandwich and looking at the surface of the two glass plates and the spacer to make sure that they were aligned.

- Then the clamp assembly was transferred to one of the casting slots in the casting stand.

➢ **Casting the gels**

- The separating gel monomer solution was prepared by combining all reagents except ammonium per sulfate (APS) and TEMED. Then the solution was deaerated and mixed after adding each reagent by swirling the container gently.
- Then a comb was placed completely into the assembled gel sandwich. With a marker pen, then a mark was placed on the glass plate 1 cm below the teeth of the comb. This would be the level to which the separating gel was poured. The comb was removed then.
- APS and TEMED were added to the monomer solution and mixed well by swirling gently. The solution was pipetted to the mark.
- The monomer solution was immediately overlaid with 1 ml of water. A steady rate of delivery of water was used to prevent mixing it with the gel.
- Then the gel was allowed to polymerize for 45 minutes to 1 hour. The water overlaying the gel was poured and the excess water was drained with strips of filter paper.
- The stacking gel monomer solution was then prepared and all reagents were combined except APS and TEMED. Then the solution was deaerated and mixed by swirling the container gently.
- Then a comb was placed in the gel sandwich.
- APS and TEMED were added to the solution and the solution was pipette down to one of the spacer until the sandwich was filled completely.
- The gel was then allowed to polymerize for 15 minutes and then the comb was removed.

- Then the gel was placed in the buffer chamber and running gel buffer was added into the chamber.

➢ **Loading the samples**

- The sample protein was taken and mixed with the sample loading buffer using a micropipette. The micropipette was inserted to about 1-2 mm from the well bottom before delivery.
- The samples were loaded in all of the wells of the gel. Now the gel was prepared for run.

➢ **Running the gel**

- Then the buffer was checked in the upper buffer chamber and ensured that they were full so that the leakage of the buffer might not occur.
- The lid was then placed on top of the lower buffer chamber and was confirmed that the connection was correct, i.e. black to black and red to red.
- The electrical leads were then attached to a suitable power pack with the proper polarity (black to black and red to red). Then the gel was run at a constant current of 30 mA.
- The electrophoresis was stopped when the tracker dye is ~ 1 cm above the end of the glass plates.

➢ **Removing and staining the gel**

- The gel was removed from the buffer chamber when the gel electrophoresis was completed.
- All four screws of the clamp assembly were then loosed and the glass plate sandwich was removed from it.
- Then one of the spacers was pushed out to the side of the plates without removing it.

Fig.-16: Coomassie Blue Gel Staining

- The spacer was then twisted gently so that the upper glass plate was pulled away from the gel.
- The gel was cut on in one side of the edge to orientate the gel.
- The gel was then removed by gently grasping two corners of the gel and was placed it in the container containing the Coomassie blue stain. It was made sure that the gel was fully submerged in the staining solution.
- The gel was then stained for 1 hour by agitating it slowly on a shaker.
- Then the gel was destained in a destaining solution for a few minutes until protein bands were visualised.
- The molecular weight of the visualised protein bands were determined by comparing them with the molecular weight markers (Fig.-17) (Roth®- Mark Standard Protein Marker, 6.5-200 kDa, Bands - 9).

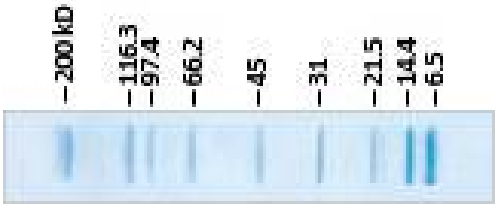


Fig.-17: Roth® - Mark standard unstained protein marker, MW: 6.5-200 kDa

3.2.7 Anticoagulation Activity of SGE

To test the anticoagulation activity of salivary gland extracts the following laboratory test was performed such as (a) Thrombin Time (TT), (b) Prothrombin Time (PT) and (c) Activated Partial Thromboplastin Time (APTT). The prothrombin time approach was preferred to measure the anticoagulation activity of salivary gland extract of the cattle ticks. Prothrombin time (PT) measures the extrinsic pathway of coagulation. They are used to determine the clotting tendency of blood. PT measures factors I, II, V, VII, and X. It is used in conjunction with the activated partial thromboplastin time (aPTT) which measures the intrinsic pathway.

3.2.7.1 Protocols of PT

3.2.7.1.1 Materials of PT

- **Patient's plasma**: Blood was collected into 31.3 g/l trisodium citrate in a concentration of 1: 9 ratios. After collection, the specimen was centrifuged at 3000 rpm for ten minutes and the supernatant that contain the desired plasma was carefully poured into a plastic tube, using a micropipette. This sample must be used within four hours after collection, unless it is frozen. So, it was kept in the deep-freeze refrigerator until tested at a later date.
- **Normal control plasma**: Freshly collected normal plasma was obtained in the same way as that of the above patient.
- **Thromboplastin IS reagents; stored at 4^0C**: This reagent can be commercially obtained or home made.

- **Calcium chloride solution 20 mM; stored at 4^0C**: Some commercial reagents had already been added to the thromboplastin reagents.

3.2.7.1.2 Methods of PT

- 50 µl of citrated human plasma was taken in an eppendorf tube. 5 µl of salivary gland extract was added to it and was incubated at 37^0C for 1 minute. 5 µl of 150 mM NaCl was used for control reaction.

Fig.-18: Sysmex CA-50 machine for PT assay

- 100 µl of thromboplastin was then added to IS reagents.
- 50 µl of 20 mM $CaCl_2$ was then added to start the reaction.
- A stopwatch was used to determine the time required for the formation of fibrin clots.
- The tube was then shaken gently every other seconds, the tube was kept under water as much as possible for maintaining the temperature. The appearance of a fibrin clot was observed and was recorded at the end-point.

- Using the patient's plasma the test was performed twice and also twice on the normal control plasma. The test was repeated if the results between two measurements differ from each other by more than 5%.
- The normal range of blood coagulation time is about 10-13 seconds (Henry, 2001). However, this depends on the thromboplastin and should be established by testing a group of healthy subjects whenever a new reagent is introduce.

3.2.8 High Performance Liquid Chromatography (HPLC)

- High Performance Liquid Chromatography (HPLC) is one of the most widely used techniques for identification, quantification and purification of mixtures of organic compounds.
- In HPLC, as in all chromatographic methods, components of a mixture are partitioned between an adsorbent (the stationary phase) and a solvent (the mobile phase).
- The stationary phase is made up of very small particles contained in a steel column. Due to the small particle size (3-5 um), pressure is required to force the mobile phase through the stationary phase.
- There are a wide variety of stationary phases available for HPLC. Such as normal phase (Silica gel), reverse phase (silica gel in which an 18 carbon hydrocarbon is covalently bound to the surface of the silica).
- C18 reverse phase column was used as stationary phase and acetonitrile, water was used as mobile phase.

3.2.8.1 Preparation of buffer A and buffer B for reverse phase HPLC

- Buffer A
  - 999 ml of de-ionized water + 1 ml of TFA.
  - Mixed by swirling.
  - Filtered through a 0.45μ PTFE membrane using a Sartorius vacuum buffer filtration system.

- Buffer B
  - 80% acetonitrile was used as buffer B.
  - 800 ml of acetonitrile was mixed with 199 ml of water and 1 ml of TFA.
  - Mixed and filtered.

3.2.7.2 Materials for reverse phase HPLC

- Slivery gland extract
- Buffer A (100% De-ionized water with 1% TFA)
- Buffer B (80% ACN with 1% TFA)
- C18 Column of 4 ml
- HPLC system

3.2.7.3 Methods for reverse phase HPLC

- Equilibration of the HPLC system and Colum
- Sample preparation and injection

3.2.7.4 Equilibration of the HPLC system and Column

- The whole system including the pump, tubing, and sample loop was washed with 100% buffer A.
- The C18 column was connected to the HPLC and first it was washed with 100% buffer B to remove any residual protein attached to it and until the base line monitored at 280nm was stable.
- Two column volumes (4ml × 2 = 8ml) of buffer B was needed to pass through the column for complete washing.
- Then the column was equilibrated with three column volume (CV, 4ml × 3 = 12 ml) of 100% buffer A.
- The flow rate was set as 1ml/min during equilibration. The equilibration was done until the base line became linear.

3.2.7.5 Gradient elution and fraction collection

- Following gradient elution programme was used in the experiment:

100% A	:	1 CV
0 - 30% B	:	1 CV
30 - 80% B	:	5 CV
80 - 100% B	:	1 CV
100% B - 100% A	:	1CV
100% A	:	1CV
20% Ethanol	:	1 CV

- Gradient elution programme

Flow rate = 1ml/min

Monitoring at 280 nm

CHAPTER 4

RESULTS

4.1 Morphological Study, dissection and collection of salivary glands

The morphological characteristics of cattle ticks (*Rhipicephalus sp*.) had been studied and the results were obtained. The adult had eight legs and was about 3 mm in length and 2 mm in breath. The length of adult female was about 9 mm and the breath was 7 mm while fully engorged with blood meal. The larvae had six legs and had a length of about 0.6 mm and 0.5 mm of breath. The nymphs had 2 mm long and 1 mm wide and had eight legs and were usually darker than larvae.

Dissection of tick was carried out in melted paraffin. Dissected 50 pairs of salivary glands were kept per centrifuge tube having 1ml phosphate buffered saline (PBS).

4.2 Extraction and Estimation of Protein from Salivary glands

The method of crude salivary gland protein extraction is mentioned in detail in the materials and methods section. Following the extraction of protein by Biuret method was used to confirm the presence as well as to estimate the amount of protein. The absorbance of 1.58 for 50 pairs of salivary gland extract of *Rhipicephalus sp.* at 280 nm was found to be equal to 2.53 mg/ml of protein as determined by Lowry method (1951) using BSA as standard.

4.3 Profiling Crude salivary gland protein on SDS-PAGE

Profiling of salivary gland protein by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done. We loaded 5 μl of crude extract and re-suspended crude extract of salivary gland of *R. microplus* and *R. annulatus* in each of the four wells of gel and run it until the tracking dye reach the bottom. The protein bands in gels were shown (Fig.-19). We used Bio Rad Unstained protein marker with a range of 6.5 Kda to 200 Kda in the experiment. A total of 6 protein bands were detected in female salivary gland extract (SGE) of *R. microplus*. The molecular mass of these protein bands were estimated at 14.4 kDa, 21.5 kDa, 33.5 kDa, 46 kDa, 66 kDa and 115, consecutively.

However, *R. annulatus* SGE did not produce any bands. This might be due to mishandling or degradation of the sample. Because, when we ran the *R. annulatus* SGE alone we did not find any bands. Later, we tried to collect the *R. annulatus* sp. but unfortunately, the attempt was failed. Therefore, we proceeded with only *R. microplus.* SGE.

We expected anticoagulant protein with a low molecular weight (within the range of 10Kda to 15 Kda). We obtained one protein band of around 14 kDa which is indicative of presence of anticoagulant protein in the crude extract of salivary gland of *R. microplus*. However, this crude protein band may contain multiple proteins as purification is still needed.

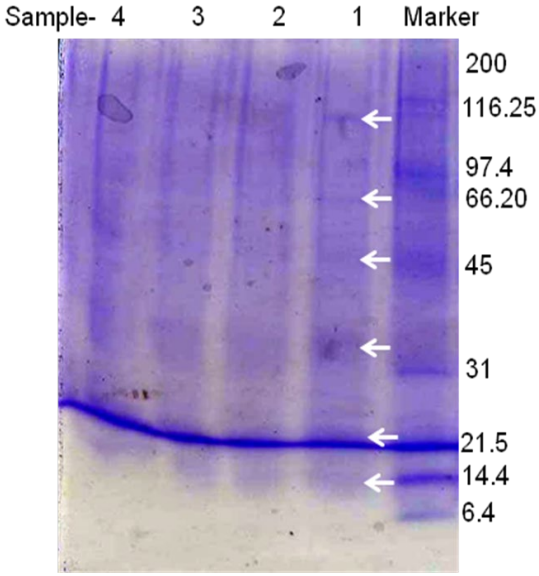


Fig.- 19: SDS-PAGE Profile of crude Salivary Gland Extract (slot-1 and slot-2 is used for *Rhipicephalus microplus* and slot-3 and slot-4 is used for *Rhipicephalus annulatus*) and a protein marker of 6.5-200 kDa was run in slot 5

4.4 Determination of anticoagulant activity of crude extract

The main objective of my research project was to determine whether the salivary gland extract of *R. microplus* and *R. annulatus* have any anticoagulant activity or not. The anticoagulation activity of the crude salivary gland extract was determined using a clinical approach namely prothrombin time (PT) assay. It is used to determine the clotting time of blood. The normal range for prothombin time is usually around 10-13 second. The results obtained in our

experiment at Popular Diagnostic Centre for this test by using Sysmex CA-50 machine are shown in table-3 as follows-

Table-3: PT assay with crude salivary gland extract of *R. microplus*

Data		Prothombin time (Sec)	Mean	Standard deviation(SD)	Standard Error (SE)	T value
Control		14	14			
Sample 5µl	1	24.6	24.54	0.4615	0.2064	2.71*
	2	24.8				
	3	25.1				
	4	23.9				
	5	24.3				
Sample 10 µl	1	27.7	27.38	0.5263	0.2354	3.05*
	2	27.3				
	3	26.8				
	4	28.1				
	5	27				

* Significance at 0.05% level of significance

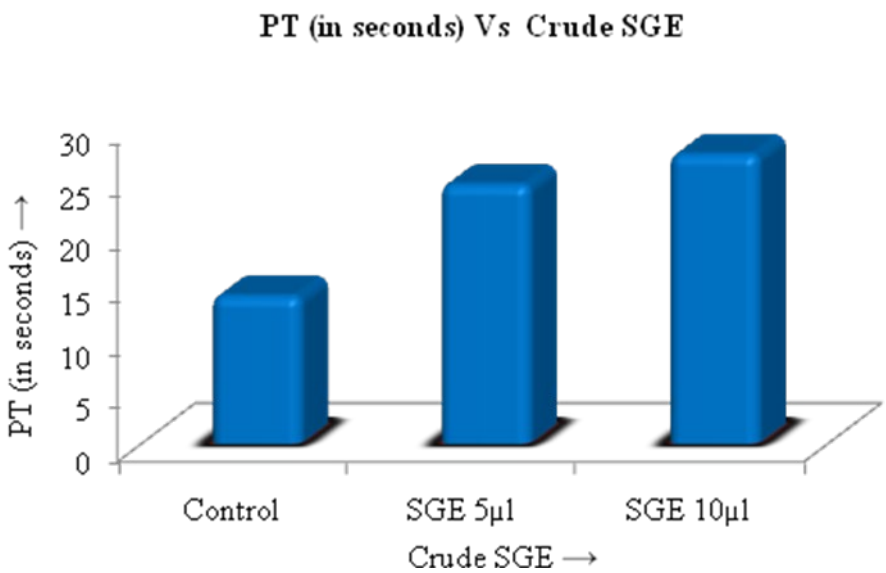


Fig.-20: PT assay with crude salivary gland extract against time (in seconds)

We used 5µl of phosphate buffered saline in order to determine the control value. When we use 5µl of crude salivary gland extract it significantly increased the prothombin time. We repeated the experiment five times which means five replication was done and the average value of the five replications was found to be 24.54 seconds with a standard deviation of 0.4615. Further, we used higher concentration (10 µl) of crude salivary gland extract to check whether it increases the prothombin time than previous or not. Similar to the method described above we took five reading and the average value was 27.38 seconds with a standard deviation of 0.5263.

It clearly indicates that the crude salivary gland extract contain anticoagulant protein. We also have shown here that the anticoagulant activity is dose dependent and that increases with the increment of protein concentration. However, in our current experiments we have used crude extract of salivary gland and hence the concentration of protein is very low. But it still shows very promising and significant anticoagulant activity. Therefore, further purification of specific anticoagulant protein is essential.

As the deviation of prothombin time from control is low, the purified protein shows more potency than the crude protein which we have done in RP-HPLC.

Dose response curve was done in our experiment. In dose response curve we found that our prepared dose response curve is nearly similar to that of standard dose response curve (Fig.-21 and Fig.-22).

4.5 Purification of crude salivary gland extract by RP-HPLC

Specific anticoagulant was purified from the crude salivary gland extract of *R. microplus* using reverse phase high performance liquid chromatography (RP-HPLC). Before the start the of RP-HPLC method crude salivary gland extracts of *R. microplus* insect were filtered through 0.22µ syringe filter and then 20 µl of crude extracts were injected in the machine by a syringe. 12 peaks were found in RP-HPLC (Fig.-23). All the peaks were tested for anticoagulant activity. Only peak 3 and peak 5 showed anticoagulant activity. Peak 3 with a retention time of 14 minutes had high anticoagulant activity where as peak 5 with a retention time of 19 minutes showed very little anticoagulant activity.

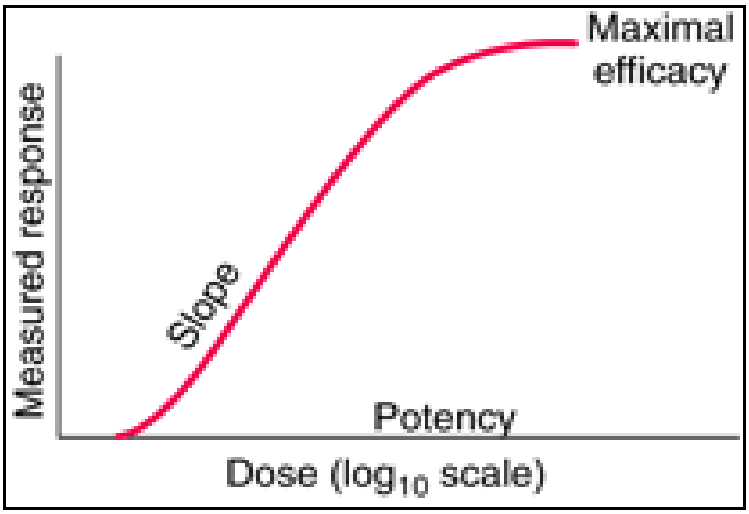


Fig.-21: Standard Dose Response Curve

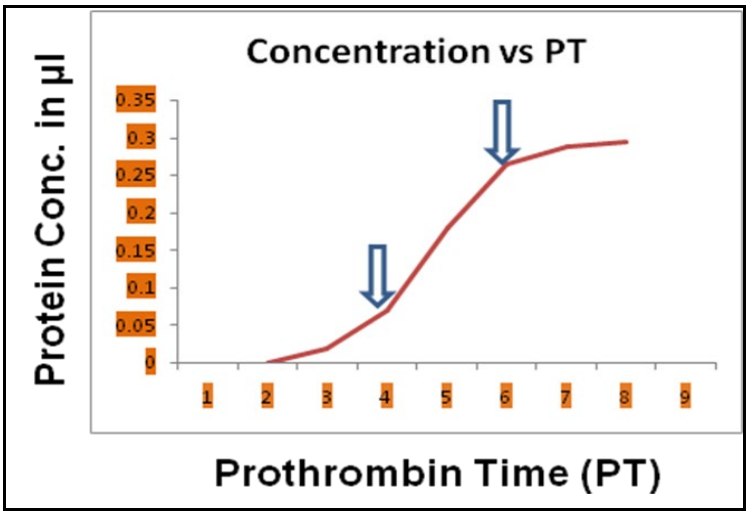


Fig.-22: Observed Dose Response Curve

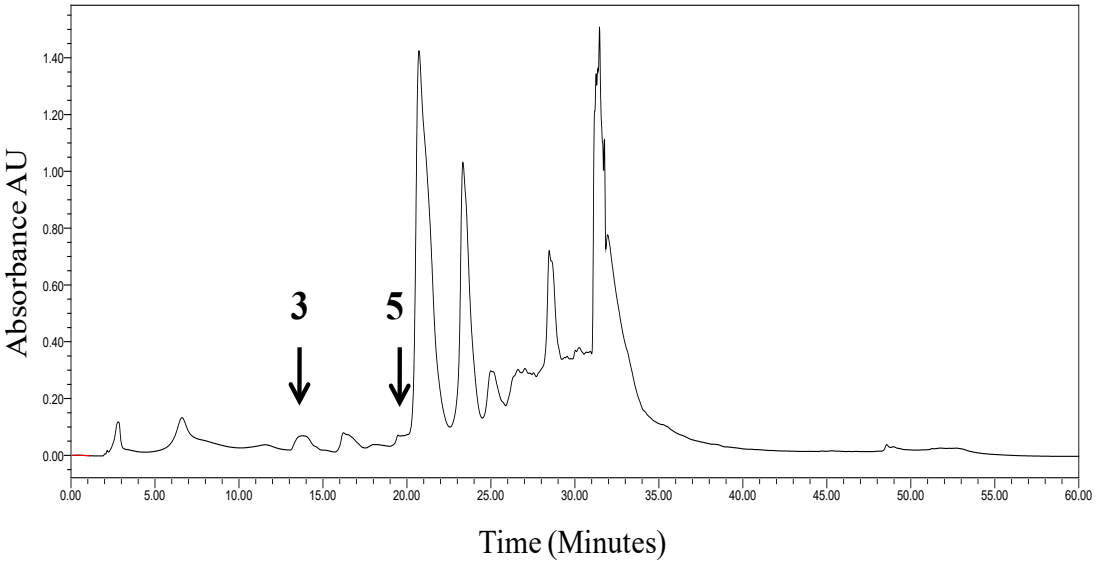


Fig.-23: RP-HPLC profile of the crude extract of *R. microplus*

4.6 Determination of anticoagulant activity of purified salivary gland extract by RP-HPLC

The anticoagulation activity of the purified salivary gland extract by reverse phase high performance liquid chromatography (RP-HPLC) was determined using prothrombin time (PT) assay. The results obtained in our experiment at Popular Diagnostic Centre for this test by using Sysmex CA-50 machine are shown in table-4 as follows-

Table-4: PT assay with purified extract of *R. microplus* by RP-HPLC

Data		Prothombin time (Sec)	Mean	Standard deviation(SD)	Standard Error (SE)	T value
Control		14	14			
Sample 5µl of peak 5	1	23.6	22.07	1.604	0.926	1.65*
	2	22.2				
	3	20.4				
Sample 5 µl of peak 3	1	29.7	32.93	3.592	2.037	1.86*
	2	32.3				
	3	36.8				

* Significance at 0.05% level of significance

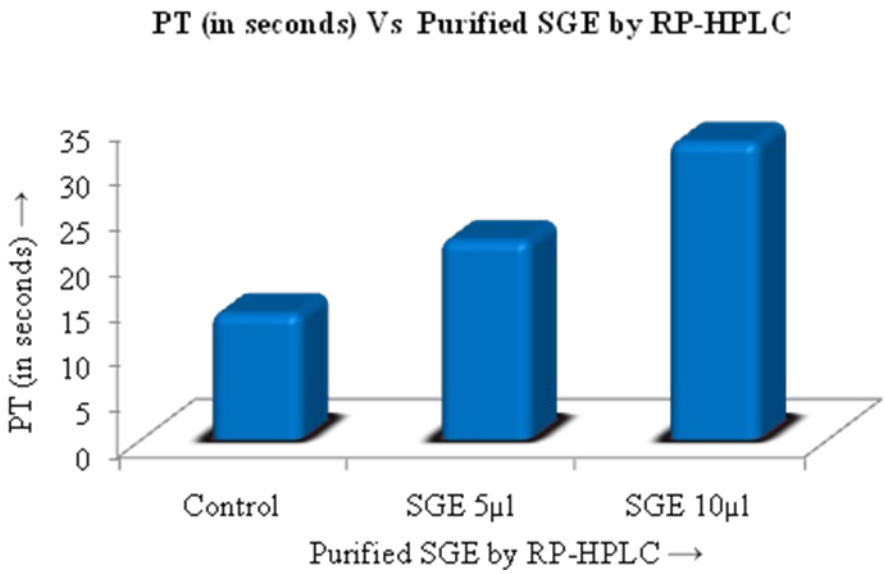


Fig.-24: PT assay with purified salivary gland extract by HPLC against time

5 µl of phosphate buffered saline was used in order to determine the control value in this case. When we use 5 µl of purified salivary gland extract of peak 3 and peak 5 we again found that it increased the prothombin time. We repeated the experiment three times. The average value of the three replications was found to be 22.27 seconds with a standard deviation of 1.604 for peak 5 and 32.93 seconds with a standard deviation of 3.592 for peak 3.

It clearly indicates that the purified salivary gland extract from peak 3 and peak 5 contain potent anticoagulant protein. The table also suggested that peak 3 contain more potent anticoagulant than peak 5.

CHAPTER 5

DISCUSSION

Blood sucking tick contains numerous anticoagulant proteins in their saliva which keep their meal in liquid form. These anticoagulant proteins have potential biopharmaceutical applications for the treatment of various thrombolytic disease or disorders.

Many prospective substances have been isolated from tick saliva, e.g. cement, vasodilators, platelet aggregation inhibitors, anticoagulants, prostaglandins, and allergens (Gauci *et al*., 1988; Jaworski *et al*., 1990; Limo *et al*., 1991; Wang *et al*., 1996 and Zhu *et al*., 1997). Several anticoagulants have also been isolated from the salivary gland of the blood sucking insects. Markwardt isolated a potent anticoagulant from leech named 'hirudin' in 2002 (Markwardt, 2002). It has been used as a commercial drug for thrombosis now a day. During the end of the year of 1998, Waidhet-Kauadio and his coworkers purified and characterized of a thrombin inhibitor from the salivary glands of a malarial vector mosquito, *Anopheles stephensi* (Waidhet-Kauadio *et al*., 1998). Another group of scientists from National University of Singapore have identified an anticoagulant peptide named 'variegin' from tropical bont tick *Amblyomma variegatum* (Cho Yeow Koh *et al*., 2007). According to their finding, tick saliva contains potent antihemostatic molecules that help ticks obtain their enormous blood meal during prolonged feeding. They isolated and characterized one of the smallest (32 residues) thrombin inhibitors found in nature.

The morphological characteristics had been studied to identify cattle ticks. Both adult and nymph had four pairs of legs while the larvae had three pairs of legs. The adult male was about 3 mm x 2 mm, the adult blood fed female was about 9 mm x 7 mm, the nymphs was about 2 mm x 1 mm and the larvae was about 0.6 mm x 0.5 mm in size. *Rhipicephalus microplus* and *Rhipicephalus annulatus* both ticks had a hard shiny scutum in their body. According to Walker and his coworkers, tick larvae have three pairs of legs and no genital aperture, nymphs have four pairs of legs and no genital aperture, females have

four pairs of legs and a large genital aperture and males have four pairs of legs and a genital aperture in the same position as the female. All ixodid ticks have a scutum or a conscutum as a hard plate on the dorsal surface (Walker *et al*., 2003).

A step wise procedure was done to isolate salivary glands from partially fed female tick and the salivary glands were crushed to extract crude salivary gland protein. Total amount of protein is estimated by Biuret method developed by Ferdinand Rose in 1833. The absorbance of 1.58 for 50 pairs of salivary gland extract of *Rhipicephalus sp.* at 280 nm was found to be equal to 2.53 mg/ml of protein as determined by Lowry method (1951) using BSA as standard. Asif and his scientist group estimated total protein from *Boophilus microplus* tick midgut and salivary glands in 2011 by Biuret method. They reconstituted the samples in PBS (pH=7.2) to achieve a final protein concentration of 10 mg/ml for their experiment (Asif *et al*., 2011). Another group of scientists quantified total protein contents from the whole salivary glands extracts of an oriental female blackfly and found out to be approximately 1.12±0.13 µg/female which is slightly lower than this experiment (Borah *et al*., 2014). According to Kazimirova and his coworkers the total soluble protein concentration of crude salivary gland extracts of female tabanid fly *Atylotus loewianus* was 65 µg/specimen or 3.25mg/50 pairs of salivary glands which supports this findings (Kazimirova *et al.*, 2002).

Crude salivary gland protein profiling was done by SDS-PAGE. 5 µl of crude extract and re-suspended crude extract of salivary glands from two different species of ticks were run in four separate wells of the gel. Six distinct protein bands were found in a range between 116.25 kDa to 6.4 kDa in our experiment. Borah and his colleagues examined salivary glands total proteins in female blackfly *Simulium indicum* in SDS-polyacrylamide gel in 2014. At least 16 major and several minor protein bands (Fig.-25) were detected in the female salivary glands. The molecular masses of these major protein bands were estimated between 69 kDa to 16 kDa (Borah *et al*., 2014).

In 1999, Tikki extracted salivary glands proteins from unfed, three days and five days fed adult hard tick *Hyalomma marginatum* marginatum and analyzed the

protein by SDS-PAGE. They estimated major protein bands in this particular tick saliva ranging from 13 kDa to 229 kDa which is agreeable with present findings (Tikki *et al*., 1999).

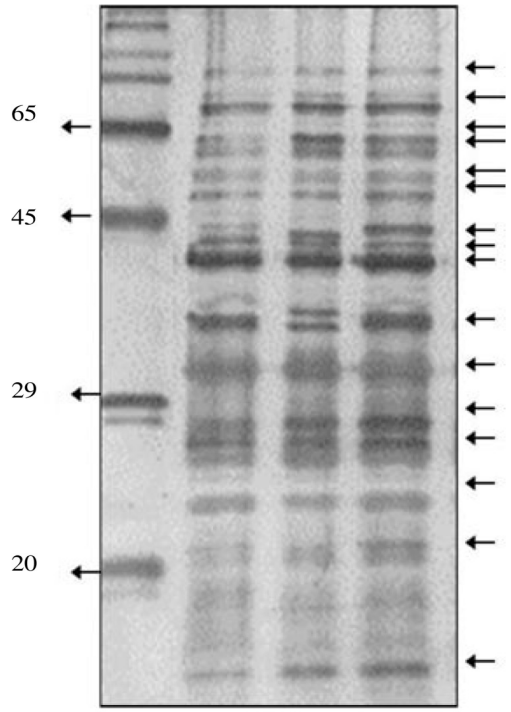


Fig.-25: SDS-PAGE profile of salivary extract of *S. indicum* (Borah *et al*., 2014)

Research revealed that, blood sucking bugs have enormous amount of anticoagulant peptides in their saliva or midgut (Jacobs *et al*., 1990; Abebe *et al*., 1994; Ribeiro *et al*., 1995; Stark *et al*., 1995 and Cappello *et al*., 1998). To test the anticoagulant activity of the salivary gland extract of two cattle ticks (*R. microplus* and *R. Annulatus*) prothrombin time (PT) assay was done. According to the current findings *R. microplus* had potent anticoagulant activity whereas *R. Annulatus* had no anticoagulant activity. This crude protein (collected from *R. Annulatus*) needs further experimentation. Some inhibitor may binds here that inhibit anticoagulant activity.

The normal range for prothrombin time is usually around 10-13 second (Henry, 2001). Using 5 µl and 10 µl of crude salivary gland extract the prothrombin time was longer than normal and that was 24.54±0.4615 seconds and 27.38±0.5263 seconds respectively. It was clearly indicated that the crude salivary gland extract of *R. microplus* contains potent anticoagulant protein. It was also shown

that the anticoagulant activity is dose dependent and that increases with the increment of protein concentration. Prepared dose response curve is nearly similar to that of standard dose response curve which supports previous findings. According to Borah, the salivary glands of the female blackfly *S. indicum* have anticoagulant activity. Anticoagulant activities against thrombin, and the extrinsic and intrinsic coagulation pathways were found in the salivary gland extract in the TT, PT and APTT assays, respectively in their experiment. It was found that salivary gland extract prolonged human plasma clotting time in a dose-dependent manner which is agreeable with current findings (Borah *et al*., 2014).

In this current experiments crude extract of salivary gland were used and hence the concentration of protein is very low. But it still shows very promising and significant anticoagulant activity. Therefore, further purification of specific anticoagulant protein is essential. As the deviation of prothrombin time from control is low, the purified protein shows more potency than the crude protein which was done in reverse phase high performance liquid chromatography (RP-HPLC). In RP-HPLC analysis several peaks were found. These peaks were tested for anticoagulant activity by PT assay. 5µl of purified salivary gland extract of each peak were tested for anticoagulant activity. Only two peaks (peak-3 and peak-5) showed significant anticoagulant activity and they increased prothrombin time. It was also observed that peak 3 had high anticoagulant activity (delay time 32.93±3.592 seconds) whereas peak 5 had very little anticoagulant activity (delay time 22.27±1.604 seconds). This peak needs further experimentation.

According to Sun and his coworkers the salivary glands of the blood sucking insect, *Rhodnius prolixus*, have an anticoagulant, prolixin-S, which was reported as a specific inhibitor of intrinsic coagulant pathway (Sun *et al*., 1996). They purified prolixin-S from the salivary glands extract by gel filtration and anion exchange HPLC by assaying prolongation of activated partial thromboplastin time (APTT). The anticoagulant factor had red color and a specific absorbance peak at 402 nm and thus it was identified as a heme

protein. Prolixin-S is a novel anticoagulant of 19,922 Da, which has no sequence homology with any other anticoagulant, reported so far.

In 2001, Kazimirova and a group of scientists' purified salivary gland extract of four horsefly species and tested their anticoagulant activity. According to them, the RP-HPLC frictions with retention time between 17-25 minutes had anticoagulant activity (Kazimirova *et al*., 2001).

We also have proteins with anticoagulant activity with similar retention time 14 minutes for peak 3 and 19 minutes for peak 5 and peak 3 showed better anticoagulant activities than peak 5.

CHAPTER 6

SUMMARY

Anticoagulants are a type of medication that reduces the risk of blood clots, including deep vein thrombosis, pulmonary embolism, atrial fibrillation and high or moderate risk of stroke. However, from the very beginning of the society research work had been carried out to find out novel anticoagulant to solve the problem of thrombotic disorders.

Haematophagus insects contain anticoagulant proteins or peptides in their saliva for their prolonged feeding. In absence of such component in saliva, the insects will starve to death. These anticoagulants have remarkable biopharmaceutical applications in treatment of various thrombolytic disorders and other therapeutic indications. Therefore, salivary gland of the blood sucking insect is a virtual gold mine for drug lead compounds for future anticoagulant drug development.

For centuries, scientists explore to acquire novel anticoagulant drug from various natural sources as well as haematophagus insects that is well documented. However, haematophagus insect from our region is still unexplored for potent anticoagulants, although we have a number of species. So it is interesting to search for anticoagulant peptides as a potential drug source from the blood sucking insects of our country.

Among different tick species found in Bangladesh *Rhipicephalus microplus* and *Rhipicephalus annulatus* are the most common species. Therefore, the current research work was done by (i) collecting salivary glands from two species of ticks, (ii) extracting protein from the salivary glands and estimating the concentration, (iii) SDS-PAGE gel running of crude salivary gland protein, (iv) checking the anticoagulant activity of crude salivary gland extract, (v) purifying of crude extracts by RP-HPLC and (vi) checking the anticoagulant activity of the purified salivary gland extract.

To carry out the work ~1000 ticks of each species were collected from different cattle farms of Rajshahi, Natore, Pabna and Chapainawabganj. After dissection

of the insects salivary glands were collected and were stored in PBS 50 pairs/centrifuge tube in -20º C.

Protein was extracted from salivary gland and concentration of protein was estimated using Lowry method (1951) which was 2.53 mg/ml for 50 pairs of salivary glands for both species. SDS-PAGE profile showed six bands within the range of 6 kDa to 115 kDa.

To determine the anticoagulant activity of the salivary gland extract prothrombin time assay (PT) was performed in Popular Diagnostic Centre, Rajshahi. Salivary gland extract of *R. microplus* showed increased clotting time in comparison to control whereas *R. annulatus* showed no anticoagulant activity. In control reaction blood clotted within 14 seconds whereas 5 µl and 10 µl of salivary gland extracts of *R. microplus* increased the clotting time to 24.54±0.4615 seconds and 27.38±0.5263 seconds respectively which was significantly high. Besides, dose response curve showed similarity to that of standard dose response curve. Therefore, present studies clearly indicated that the salivary gland extract of *R. microplus* has great potential as an anticoagulant agent.

Further purification of specific anticoagulant from the crude salivary gland extract of *R. Microplus* was carried out using reverse phase high performance liquid chromatography (RP-HPLC). Picks obtained in RP-HPLC were tested for anticoagulant activity by PT assay. Using 5 µl of each sample, only two peaks (peak-3 and peak-5) showed significant anticoagulant activity then earlier. Peak 3 with a retention time of 14 minutes had high anticoagulant activity (delay time 32.93±3.592 sec.) where as peak 5 with a retention time of 19 minutes had very little anticoagulant activity (delay time 22.27±1.604 sec.). Thus, Pick 3 can be developed and further optimized as anticoagulant for pharmaceutical use.

However, further research is needed to purify the precise protein responsible for anticoagulant activity from the crude salivary gland extract or the mixtures.

CHAPTER 7

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