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Anticoagulant activity of salivary gland extract of a haematophagus insect, *Rhipicephalus microplus* from Bangladesh

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Abstract

Blood sucking insect contains anticoagulant proteins or peptides in their saliva. In absence of such component in the saliva, insects will starve to death. These anticoagulants have remarkable biopharmaceutical applications in treatment of various thrombolytic disorders and other therapeutic indications. Therefore, these insects and their salivary gland is a virtual gold mine for drug lead compounds for future anticoagulant drug development. A number of research groups are currently involved in research on different haematophagus insects around the world. However, so far haematophagus insect of our region is totally unexplored. Therefore, it is interesting to search for anticoagulant activity of salivary gland extract (SGE) as a potential anticoagulant drug source from the blood sucking insects of our country. Among different tick species in Bangladesh *Rhipicephalus microplus* is the most common one. In the current project we (i) collected salivary glands from *R. microplus*, (ii) extracted protein from the salivary glands and (iii) examined anticoagulant activity of the salivary gland extract using prothrombin time (PT) assay. In comparison to control (14 sec), the salivary gland extract shows increased clotting time of 14.62 second, 16.42 second, 22.74 second, 26.18 second, 26.98 second and 27.04 second respectively in 2.5 µl, 5 µl, 7.5 µl, 10 µl, 12.5 µl and 15 µl concentration of SGE. Thus, *R. microplus* from Bangladesh has potential as a source of anticoagulant lead molecule. Further experimentation on isolation of anticoagulant molecule is in progress.

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Introduction

Whenever any vascular injury occurs the immediate result is bleeding. However, the bleeding is stopped within short period of time by the mechanism of coagulation of blood followed by repair of the haemostatic system. Precise control over blood clotting is important for survival of any animal. If bleeding cannot be stopped at proper time it can endanger life. On the other hand unwanted clot formation (for example, stroke, DIC) can also be fatal. To treat different bleeding disorder we need either procoagulant or anticoagulant molecule.

Anticoagulants reduce blood clotting and thus prevent deep vein thrombosis, pulmonary embolism, myocardial infarction and stroke. A number of insects and animals suck blood for their survival. Mosquitoes, ticks, bedbugs, flies, butterflies, leeches, bats are some examples of blood sucking creatures. Blood sucking insects produce multiple biologically active compounds in their salivary gland. But, all of them have one thing in common: they contain anticoagulants which they apply to the host to create an optimal environment for their extended feeding time (Mans and Neitz 2004; Ribeiro 1987).

Coagulation control of sucked blood may be the most important concern for the hematophagous insects since it occurs in the earliest stage of their invasion. During their feeding and even after feeding the haematophagus insects need to keep the sucked blood in liquid form in order to keep their feeding tube clear.

This is only possible if their saliva contain proper anticoagulant. For this reason, scientists are looking for potent and safer anticoagulant protein from the hematophagous insects. A number of anticoagulants have been isolated from the salivary gland of the blood sucking insects which they use during feeding (Basanova *et. al.* 2002; Zavalova *et. al.* 2002). The most famous anticoagulant protein is 'hirudin' which is used as a commercial drug for thrombosis was isolated from leech (Markwardt 2002). There are also many kinds of anticoagulant molecules identified from different insect species (Mans and Neitz 2004; Mulenga *et. al.* 2000).

Though a number of works has been carried out on tick variety, a very little work is done on *R. microplus* (Ricci *et. al.* 2007). However, there is no report of the anticoagulant activity of the saliva of this tick in Bangladesh as well as from our sub continental region. Due to geographical variation the composition of saliva including its anticoagulant may vary significantly. Therefore, it is interesting and important to work with this tick which may aid in future drug development.

Ticks belong to two families namely Argasidae and Ixodidae of class Arachnida that are ectoparasite in nature. The members of the tick family Ixodidae are commonly known as hard ticks, and those of Argasidae, the soft ticks. The cattle tick is an external parasite, and is regarded as a significant economic pest of the most cattle industry.

The cattle tick is widely distributed in Central and South America, parts of the southern USA, Africa, Asia, and northern Australia. In our country *Haemaphysalis bispinosa* (cattle, goats, and dogs), *R. microplus* (cattle, goats), and *R. sanguineus* have been recorded in the hilly areas of Chittagong (Fuehrer *et al.* 2012). Therefore, the current project was under taken to evaluate the potentiality of the anticoagulant proteins present in the salivary gland of cattle tick- *R. microplus* collected from three northern districts of Bangladesh to use it as a commercially viable anticoagulant drug source.

Materials and methods

Collection of cattle tick

Cattle ticks were collected from the dairy animals of local cattle farms of Rajshahi, Natore and Chapainawabganj, three northern districts of Bangladesh. Partially fed adult female ticks were preferred for collection by hand picking method. After collection, ticks were kept in plastic vials in normal temperature. Sufficient aeration was maintained by closing the tubes using cheesecloth.

Dissection and Isolation of Salivary Glands

A step-by-step procedure for the dissection and isolation of the tick (*R. microplus*) was followed using the protocol described by Edwards with minor improvisation (Edwards *et. al.* 2011). The dissection process was performed under stereomicroscope. 100 pairs of isolated salivary glands were isolated, preserved in 1ml PBS solution and were stored at -27°C.

Protein Extraction from Crude Salivary Gland Extract

The stored salivary glands were thawed to room temperature for 5 minutes and were centrifuged at 10,000 rpm for 30 seconds in a 1.5 ml micro-centrifuge tube. Supernatant (PBS solution) was carefully removed using a micropipette. Liquid nitrogen was added to the tube and the glands were crushed to homogeneity using a homogenizer. Subsequently, 200 µl of 150 mM NaCl solution was added to the centrifuge tube and was incubated at room temperature for 5 minutes.

The mixture was spun for 10 min at 10,000 rpm. The supernatant was taken carefully using a micropipette without disturbing the pellet in an autoclaved micro-centrifuge tube.

This supernatant is termed as salivary gland extract (SGE) in rest of the manuscript and was used in the subsequent experiments. The pellet was re-suspended in 200 µl of 150 mM NaCl solution and same procedure was repeated. SGE were stored at -30° C.

Estimation of total protein content

Total protein present in the salivary gland was estimated using Biuret method. Protein standard was taken in one test tube and five different concentrations of sample proteins (SGE) were taken in five test tubes. Volume of all the test tubes was made 1 ml using de-ionized water. A tube containing 1 ml de-ionized water was used as blank. 4 ml of biuret reagent were added in all the test tubes, mixed thoroughly by vortexing and incubated at 25°C for 30 minutes. Subsequently, absorbance at 546 nm wavelengths was taken for blank, standards and samples.

Anticoagulation Activity of SGE

The anticoagulant activity of the salivary glands extract was tested using prothombin time (PT) assay (Kini and Banerjee 2005). Blood were collected from healthy volunteers upon signing the consent form. For performing prothombin time assay 45 µl of citrated human plasma was taken in two 1.5 ml micro centrifuge tubes. 5 µl 150 mM NaCl and 5 µl of crude salivary glands extract were added separately. Both tubes were incubated at 370C for 1 minute. Following incubation, 100 µl of thromboplastin IS reagent was added to each of the reaction tubes. Finally, 50 µl of 20 mM $CaCl_2$ was added to start the reaction.

Results

Morphological Study, dissection and collection of salivary glands

The morphological characteristics of cattle tick were studied. The adult tick has eight legs and it is about 3 mm in length and 2 mm in breadth. While an adult female tick is fully engorged with blood meal it enlarges both in length and breadth. The length of it then becomes ~9 mm and while breadth becomes ~7 mm.

The larva has six legs and has a length of about 0.6 mm and breadth is about 0.5 mm. The nymphs were around 2 mm long and 1 mm wide with eight legs and were usually darker in color than larvae. Dissection of tick was carried out in melted paraffin. Dissected salivary glands were kept in PBS in 100 pairs (Figure 1).

Extraction and estimation of protein from salivary glands

Following the extraction of protein, Biuret method was used to confirm the presence as well as to estimate the amount of protein. The value of Biuret test reading was 0.013 and the calculated amount of protein was 2.53 mg/ml. The result of biuret test is represented in Table 1.

Table 1. Amount of protein estimation using biuret test. In the estimation of total protein content de-ionized water was used as blank. 4 ml of biuret reagent were added in all the test tubes and incubated at 25°C for 30 minutes. Absorbance was taken at 546 nm wavelength for blank, standards and samples.

Sample	Volume of BSA/Protein (ml)	Volume of Distilled water(ml)	Volume of Biuret reagent(ml)	Absorbance at 546 nm	Average absorbance of sample nm	Concentration (mg/ml)	Average concentration of sample (mg/ml)
Blank	0.0	1.0	4	-		-	
Standard	1.0	-	4	0.411		79.99	
Sample-1	1.0	-	4	0.013		2.53	
Sample-2	1.0	-	4	0.010		1.95	
Sample-3	1.0	-	4	0.015	0.013	2.92	2.53
Sample-4	1.0	-	4	0.014		2.72	
Sample-5	1.0	-	4	0.013		2.53	

Determination of anticoagulant activity of the salivary gland extract

The main objective of the present work was to determine whether the salivary gland extract of *R. microplus* has significant anticoagulant activity or not. The anticoagulant activity of the crude SGE was determined using a clinical approach namely prothrombin time (PT) assay. The normal range for clotting time is usually around 10-13 second. The results obtained in our experiment were found to be significantly higher compared to the normal clotting time in response to different concentrations of SGE are shown in figure 2.

5µl of phosphate buffered saline was used in order to determine the control value. When 2.5µl of crude salivary gland extract was used, it increased the clotting time.

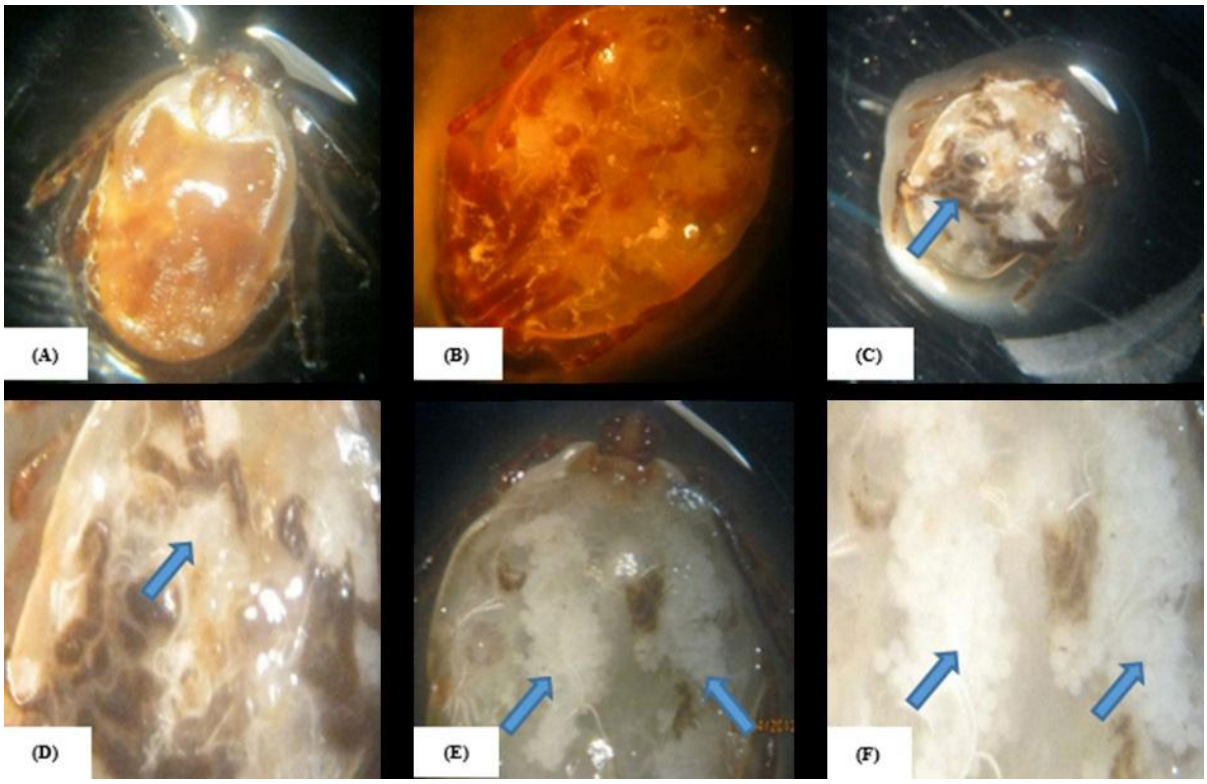

Fig. 1. Dissection of *R. microplus*. (A) Female tick rinsed with PBS after anesthetization, (B) Removed scutum by scalpel, (C) Arrow showing complete gut, (D) Arrow indicates salivary gland near the midgut, (E) Arrow indicates clear grape like salivary glands, (F) Arrow indicates, salivary glands close up.

The experiment was repeated five times and the average clotting time of the five replications was found to be 14.62 second. Further, higher concentrations (5 µl, 7.5 µl, 10 µl, 12.5 µl and 15 µl) of crude salivary gland extract were used to check whether it increases the clotting time or not. Similarly five readings were taken and the average clotting time was 16.42 second, 22.74 second, 26.18 second, 26.98 second and 27.04 second respectively. Our work clearly indicates that the crude salivary gland extract contains anticoagulant protein. It is also shown here that the anticoagulant activity is dose dependent and that increases with the increment of protein concentration. Although, in the current experiments crude extract of salivary gland was used and hence the concentration of protein was very low, but it still shows very promising and significant anticoagulant activity.

Discussion

For decades, research is being carried out to find out novel anticoagulant drug to solve the problem of thrombotic disorders in human and other animals. Recently the scientists from National University of Singapore have identified an anticoagulant peptide from tropical bont tick (Koh *et al.* 2007). 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. Scientists of Mie University, Japan extracted an anticoagulant protein, Prolixin-S from *Rhodnius prolixus* which is a specific inhibitor of intrinsic blood coagulation pathway (Sun *et al.* 1996).

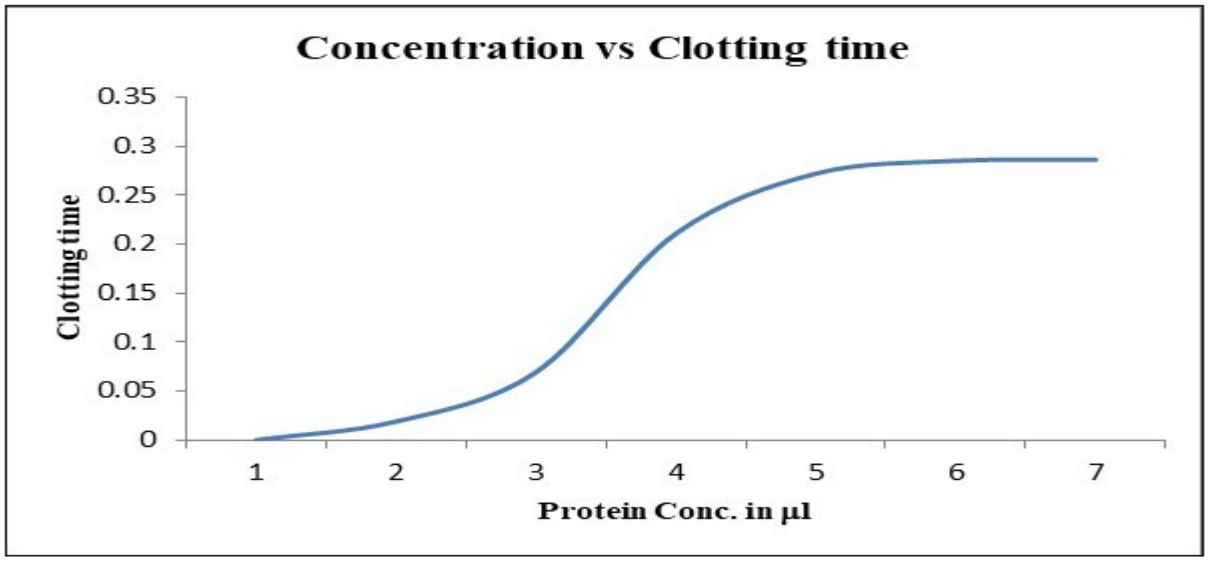

Fig. 2. Anticoagulant Activity of *R. microplus* SGE is shown through dose response curve. With increasing concentration of the SGE the coagulation time increases in a dose dependent manner. In here 2.5µl, 5µl, 7.5µl, 10µl, 12.5µl and 15µl concentration of SGE were used.

The scientist of Arizona University, isolated nitrophins, a salivary gland protein from the blood sucking insect *Rhodnius prolixus* which inhibits platelet aggregation (Weichsel *et. al.* 1998). In recent year, a novel thrombin inhibitor was isolated from the gut of *R. microplus* (Ricci *et al.* 2007). Liao and his co-workers isolated another thrombin inhibitor Hemalin (20 Kda) from the midgut of *R. microplus* (Liao *et al.* 2009). Very recently researchers (Soares *et al.* 2012) isolated yet another thrombin inhibitor Boophilin from *R. micoplus* midgut. So, it reveals that *R. micoplus* is a very rich source of anticoagulant molecule. But, in the current study crude protein from the cattle tick salivary gland was extracted instead of midgut and has clearly demonstrated that the SGE also has significant anticoagulant activities.

Conclusion

Although a number of anticoagulant drugs such as Hirudin, Heparin, Warfarin, Aspirin, Coumarin, Warfarin, Enoxaparin and Bivalirudin are currently available in market but they are relatively expensive due to high production cost and they are fairly narrow in their effectiveness with side effect.

From the present research project it can be expected that anticoagulant protein from the salivary gland extract of *R. microplus* will be more potent, safe to use, promising for the future drug development and which will be cost effective.

This is the first report of any anticoagulant activities of blood sucking insect from Bangladesh.

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