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In vitro effects of *Rhipicephalus* (*Boophilus*) *microplus* tick's salivary gland inhibitory proteins/peptides on bovine platelet aggregation and adhesion

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Abstract
Ticks are of vast importance in veterinary sciences due to their ability to transmit a wide variety of haemoprotozoan parasites such as *Theileria*, *Babesia* etc in domestic animals. Successful feeding of ticks on their host relies on a set of bioactive chemicals concentrated in their highly specialized salivary glands. Salivary chemicals of ticks have the ability to inhibit platelet aggregation in host animal via inhibition of their haemostatic mechanism. The present study targets isolation, characterization of tick saliva contents to understand the biochemical mechanisms of platelet inhibition by the inhibitory tick's salivary proteins/peptides. Accordingly, hundred pairs of tick salivary glands were dissected out and an extract was prepared by homogenizing them in HEPES buffer. Proteins in fraction nos. 22, 23, 25, 27, 31, 36, 38, 39 and 51 obtained by gel filtration chromatography were found to inhibit thrombin induced platelet aggregation and platelet adhesion to collagen when compared to agonist thrombin. The platelet adhesion inhibition by the proteins/peptides in fractions was significantly higher than agonist and lower than antagonist which indicates inhibition of receptors GP1b, GPVI and integrin $\alpha 2\beta 1$, which are previously reported to be responsible for platelet aggregation. Result indicates inhibition of platelet aggregation in host blood can be possibly achieved through inhibition of platelet activating receptors by inhibitory peptides present in tick salivary gland secretions. Exploration of these anti-platelet aggregating proteins/peptides can be further exploited for raising vaccine to control ticks and ticks borne diseases and also for therapeutic purposes.

Keywords: Anti-platelet aggregating proteins/peptides, gel filtration chromatography, *Rhipicephalus* (*Boophilus*) *microplus*, platelet adhesion, salivary gland, thrombin

1. Introduction

The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is an obligate hematophagous parasite and is considered as one of the potentially most harmful bovine arthropod [9]. Infestation of ticks on animal can cause irritation, depression of immune function, damage to hides, production loses and transmission of pathogenic micro-organisms [3, 1, 17] causing diseases like bovine babesiosis, tropical bovine theileriosis and anaplasmosis in animals resulted into huge monetary loss. In India, the control cost of ticks and tick-borne diseases in the dairy sector has been estimated at the tune of 498.7 million USD per annum [11].

Like all hematophagous parasite, ticks saliva also contains variety of bioactive molecules which they inject into host blood to avoid activation of host haemostatic, inflammatory and immunological responses [7]. Haemostasis is a complex mechanism that controls blood loss from damaged blood vessels through a series of physiological events resulting into formation of a platelet plug, fibrin clot and fibrinolysis leading to termination of blood loss [7]. Thrombin plays a central role in haemostasis. It hydrolyses fibrinogen to form the fibrin-clot, which induces platelet aggregation and activates other coagulation factors to reinforce the coagulation cascade. Thrombin induced platelet aggregation depends on the interaction among thrombin reserves and the substrates molecules available on platelet membrane, namely, platelet activating receptors-1 (PAR-1) and platelet activating receptors-4 (PAR-4) [14, 8]. Thrombin inhibitors are predominantly used in prophylaxis and treatment of acute myocardial infarction, deep venous thrombosis, pulmonary embolism and also arterial re-occlusion [6]. Scarce information is available regarding effects of tick salivary peptides on platelet aggregation.

The present study was aimed to isolate, characterize and explore the mechanism of action of inhibitory peptides and their potential use in development of novel anti-thrombotic drug as well as in exploring these molecules to raise anti-tick vaccine. In addition, it will also contribute to our understanding of tick-host interaction during blood feeding on host [10].

2. Materials and Methods

2.1 approval

Research was conducted after due approval from Institutional Animal Ethics Committee.

2.2 Sample collection

Rhipicephalus (*Boophilus*) *microplus* female ticks were collected from villages around Hisar district of Haryana and identified as per the key given by Miranpuri and Gill [12]. Ticks were washed with normal saline.

2.3 Collection of ticks salivary gland

Ticks were immobilized on a petridish with their dorsal surface upward using glue. The petri plates were kept on ice. A hundred pairs of tick salivary glands were dissected out under a stereoscopic microscope (Magnus MSZ-TR) by incising along the dorsal-lateral margin of ticks using fine scalpel blade and then transferred into HEPES saline buffer, pH 7.0. Salivary glands were stored in liquid nitrogen till further analysis.

2.4 Preparation of salivary gland extract

Dissected out salivary glands were homogenized using tissue homogenizer (T10 basic ULTRA-TURRAX®, India) in HEPES saline buffer, pH 7.0 under cold conditions to prepare salivary gland extract. Extract was then centrifuged at 12,000 X g for 7 min at 4°C and supernatant was removed which was then filtered through Millex-GV Syringe Filter Unit (25 mm PVDF 0.22 µm Sterile with Vent). The resultant filtrate was diluted to 2 ml with 50 mM Tris-Cl, pH 8.3.

2.5 Isolation of salivary anti-platelet aggregating factors from the salivary glands

The filtrate was applied to a Sephacryl S-200 gel filtration column (1 cm × 60 cm) equilibrated with 50mM Tris-HCL, pH, 7.5 with 100mM KCL and eluted with 40 mM Tris-Cl, pH 7.5. A total of 120 fractions each of 1.5 ml were collected. The column was calibrated with molecular-weight markers from Sigma (alcohol dehydrogenase, 150 kDa; albumin, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome C, 12.4 kDa; and the void volume determined with Blue Dextran, 2000 kD). The approximate molecular weights of proteins were determined using a standard curve of V_e/V_o against log molecular weight.

2.6 Total protein estimation in fractions

Total protein concentration in each fraction was estimated by Bradford [2].

2.7 Preparation of platelets

Blood sample was collected by using 0.1M trisodium citrate as anticoagulant in 9:1 from buffalo calves maintained in the Department and centrifugated at 1000rpm to obtain platelet rich plasma (PRP). Then PRP so obtained was centrifuged at 4000rpm to get the platelet pellet. Wash platelet pellet with Tyrode buffer 'A' (with EGTA) twice and then the final pellet was resuspended in the Tyrode buffer 'B' (without EGTA), in a volume adjusted to give an OD of 0.15 at 650 nm.

2.8 Assay for platelet aggregation

Effect of different isolated protein fractions on platelet aggregation was measured as per the method followed by Francischetti *et al.* [4]. Platelets were incubated with Gly-Pro-Arg-Pro (1mM) amide as antagonist and isolated protein fractions for 10 min at 37 °C in 96 well flat bottom plate. Thrombin (0.5nM) is used as agonist to initiate aggregation. Changes in platelet aggregation was monitored at 650nm at every 5 minute interval for 20 minutes using ELISA plate reader.

2.9 Assay for platelet adhesion

Platelet adhesion assay was performed by using fractions having platelet aggregation inhibition activites as per the method followed by Francischetti *et al.* [4]. Coating of 96-well microtitre plates with 2 µg of collagen prepared in phosphate-buffered saline, pH 7.4 was done. Keep the plate for overnight at 4°C. Platelets were labelled with calcein (2.5µM) by incubating for 30 min in dark with gentle mixing. Labelled platelets were taken in collagen coated wells of the microtitre plate and then incubated with Gly-Pro-Arg-Pro amide (1mM) as antagonist and fractions having platelet aggregation inhibitory activity for 10 min at 37 °C. Thrombin (0.5nM) is used as agonist to initiate aggregation. Fluorescence emitted by adhesion of platelets to collagen was measured using excitation wavelength of 492 nm and an emission wavelength of 535 nm by using Biotech Synergy-2.

2.10 Statistical analysis

The data was subjected to standard error of means (SE) and one way ANOVA test using SPSS statistical software for statistical significance [16].

3. Results & Discussion

A total of 120 fractions from *Rhipicephalus* (*Boophilus*) *microplus* salivary gland extract were collected by gel filtration chromatography. Out of these 120 fractions, fraction nos. 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 51, 52, 54, 57, 60, 63, 64, 65, 68, 73, 74, 75, 79, 80, 87 and 112 were found to have total protein concentration of 6.9, 10.1, 48.1, 61.3, 48.7, 33.6, 39.3, 39.7, 39.8, 40.8, 35.1, 41.3, 40.9, 28.7, 20.8, 21.5, 10.1, 9.2, 6.7, 0.88, 16.9, 19.8, 0.5, 16.4, 20.7, 49.8, 6.9, 2.1, 4.7, 6.8, 13.7, 12.5, 17.1, 21.9, 15.4 and 9.7 µg/ml respectively Then the effects of proteins/peptides present in above mentioned fractions on platelet aggregation inhibition were studied and found that platelet aggregation inhibition in fraction nos. 22, 23, 25, 27, 31, 36, 38, 39 and 51 were similar to that of Gly-Pro-Arg-Pro amide induced platelet aggregation inhibition (Figure 1). This Gly-Pro-Arg-Pro amide is already known to inhibit the aggregation.

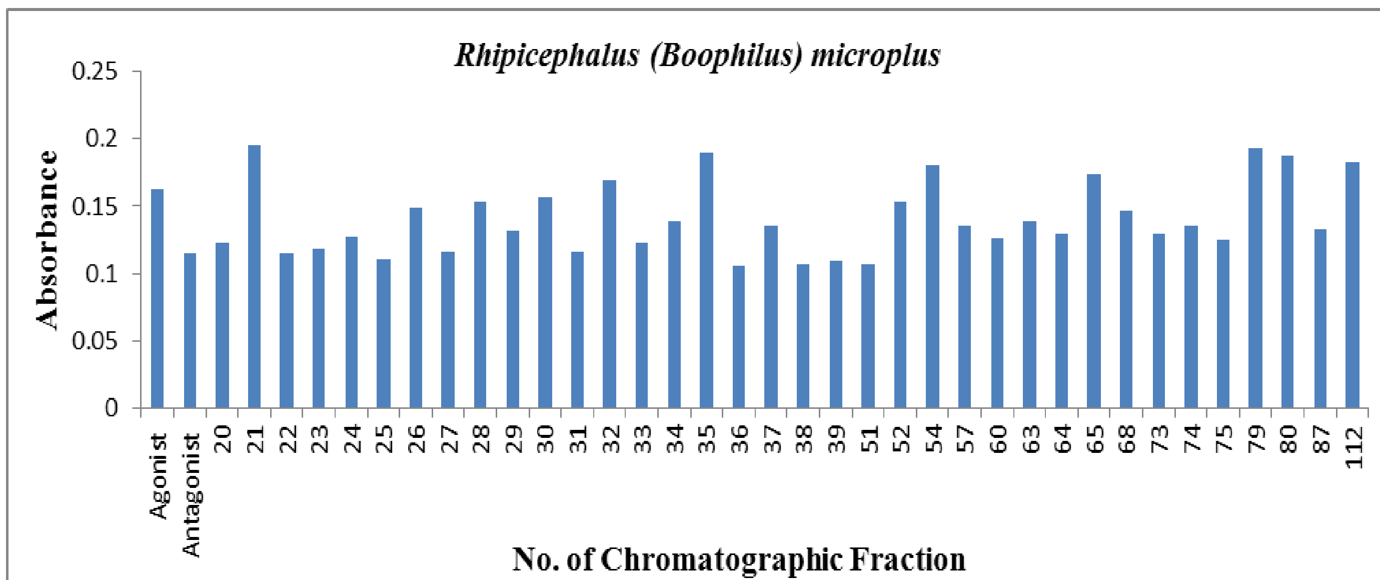


Fig 1: Effects of *Rhipicephalus* (*Boophilus*) *microplus* salivary gland protein fractions on Bovine platelet aggregation.

Percent platelet aggregation inhibition due to salivary gland proteins/peptides is presented graphically in Figure 2. The data regarding the individual fractions having platelet aggregation inhibitory activities i.e. fraction nos. 22, 23, 25, 27, 31, 36, 38, 39 and 51 is also presented in figure 3A-I. Inhibitory activities in the above mentioned fractions were nearly similar to that of antagonist (Gly-Pro-Arg-Pro amide) and much higher than agonist (thrombin). However, inhibitory activities in fraction nos. 36, 38 and 51 (Figure 3F, 3G and 3I) have slightly higher than antagonist (Gly-Pro-Arg-Pro amide) indicating platelet aggregation inhibition is due to the proteins/peptides present in these fractions.

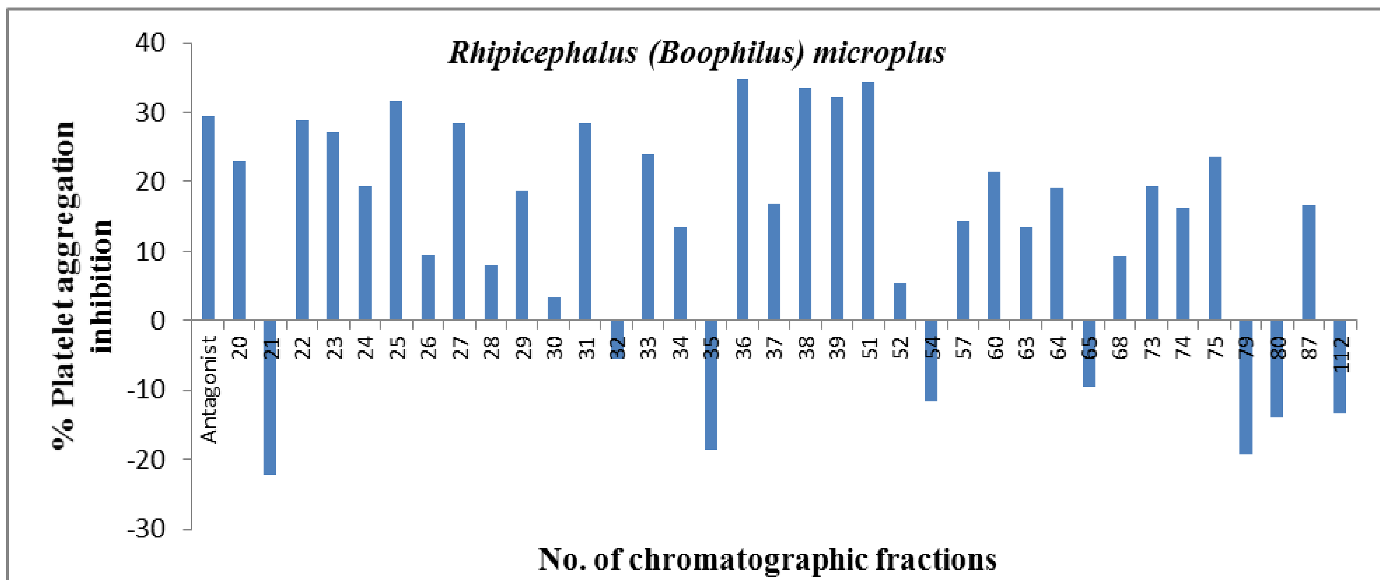
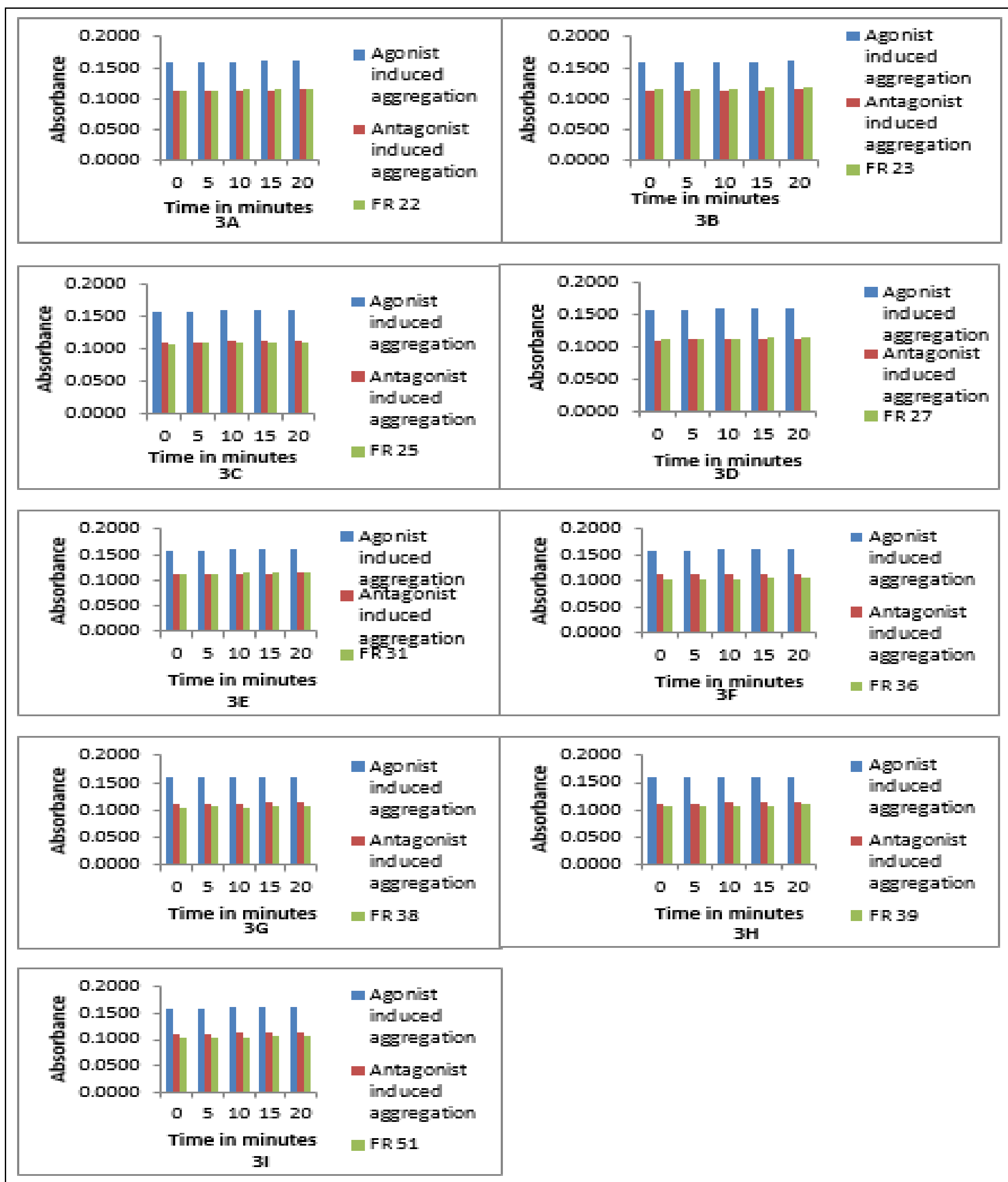


Fig 2: Effects of *Rhipicephalus* (*Boophilus*) *microplus* salivary gland protein fractions on Bovine platelet aggregation (%)



FR- No. of Gel exclusion chromatographic fractions.

Fig 3(A-I): Effects of *Rhipicephalus* (*Boophilus*) *microplus* salivary gland individual protein fractions on Bovine platelet aggregation inhibition after stimulation with thrombin (0.5nM).

Thrombin plays a vital role in blood coagulation by promoting platelet aggregation and by converting fibrinogen to form the fibrin clot in the final step of the coagulation cascade. Thrombin inhibitors depends on the interaction among thrombin exosites and the substrates on platelet membrane, namely platelet activating receptors-1 and platelet activating receptors-4 [14, 8]. The inhibitory protein/peptide present in salivary gland fractions in the study might be inhibiting host's hemostatic mechanism by inhibiting thrombin induced platelet aggregation which helps in feeding of ticks. Therefore, thrombin is an important target in anti-thrombotic therapy [20, 21]. Similarly, IxscS-1E1 is a blood meal-induced serine protease inhibitor from *I. scapularis* (Ixsc) tick saliva and was found to inhibit 23.4% thrombin induced aggregation [19]. BarI, isolated from the venom of Bothrops barnetti found to dissolves fibrin clots made either from purified fibrinogen or from whole blood [15]. Similarly, BmGTI isolated from the gut of *Boophilus microplus* ticks inhibits thrombin-induced platelet aggregation [18]. So the possible mechanism of thrombin induced aggregation inhibition isolated from *Rhipicephalus* (*Boophilus*) *microplus* can be through the inhibition of PAR-1 and PAR-4 or through exosite inhibition.

Normally when blood vessel get injured then platelets adhere to collagen through GP1b, GPVI and integrin α2β1 receptors [5] present on platelet surface resulting into their activation and aggregation. Therefore, platelet adhesion assay was performed to see the effects of proteins/peptides present in above mentioned fractions on platelet adhesion to collagen in order to explore the mechanism of action of platelet aggregation inhibition. The results showed platelet adhesion to collagen inhibition activities in fraction nos. 22, 23, 25, 27, 31, 36 and 51 ranged from 40178.0±146.6 to 43378.2±9938.3 which was significantly higher than agonist thrombin

(44309.5± 88.2) and lower than the antagonist Gly-Pro-Arg-Pro amide (39342.0±224.2). On the other hand, Fraction no. 38 and 39 showed inhibitory platelet adhesion activities similar to that of antagonist 39230.0±207.4 and 39459.0±21.3 (Table 6) suggesting the inhibition of platelets aggregation through inhibition of receptors GP1b, GPVI and integrin α2β1. The effects of peptides on platelet adhesion inhibition are shown graphically in Figure 4. Platelet adhesion inhibitors from other species have also been reported like from saliva of blood-sucking triatomine bug *Triatoma pallidipennis* contain a factor named pallidipin which specifically inhibits collagen-induced platelet aggregation but had no effect on platelet adhesion to collagen indicating that platelet-collagen interaction, the adhesion of platelets, and the shape change, was not inhibited by pallidipin but blocks the transduction of a signal leading to the activation of platelets [13]. Similarly calin was isolated from the saliva of the medicinal leech *Hirudo medicinalis*, found to be potent inhibitor of collagen mediated platelet adhesion and activation in addition to inhibition of binding of von Willebrand to coated collagen [22]. Exploration of characteristics and mechanisms of action of these peptides can be useful in therapeutics as well as for raising anti-tick vaccine for tick control.

Table 1: Changes in fluorescence emitted (Mean±SE) by platelet adhesion in the presence of agonist, antagonist and salivary gland fractions of *Rhipicephalus* (*Boophilus*) *microplus.* (n=4)

Species	Agonist induced platelet adhesion	Antagonist induced platelet adhesion inhibition	Salivary gland Fraction no.								
			22	23	25	27	31	36	38	39	51
Rhipicephalus (*Boophilus*) *microplus*	44309.5± 88.2[a]	39342.0± 224.2[e]	42384.5± 355.1[bc]	43378.2± 938.3[ab]	40178.0± 146.6[de]	41201.7± 379.6[cd]	41627.5± 612.4[cd]	42311.2± 488.7[bc]	39230.0± 207.4[e]	39459.0± 21.3[e]	41569.2± 161.1[c]

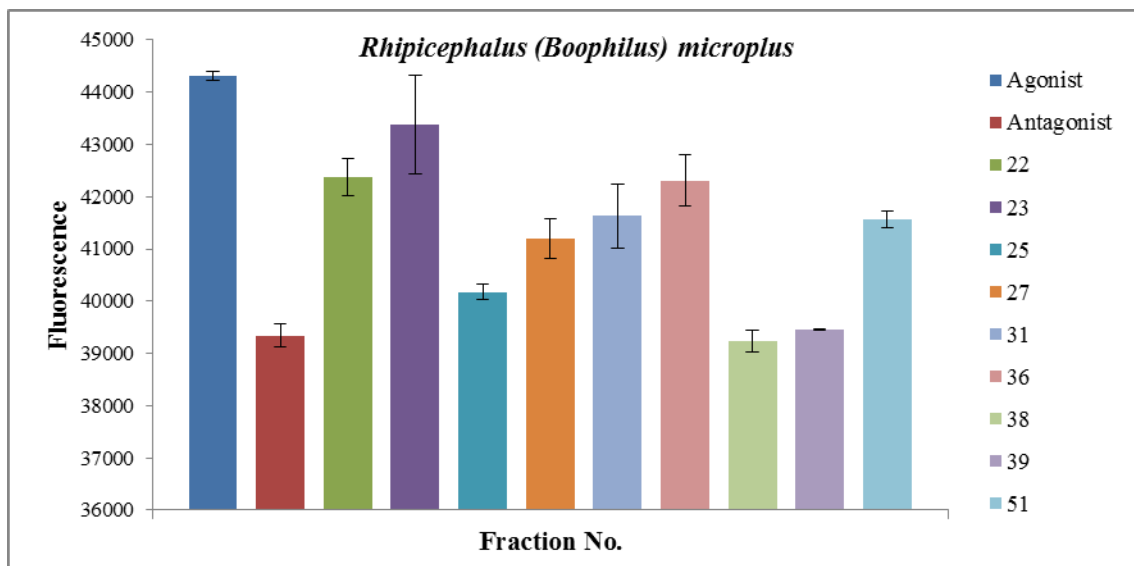


Fig 4: Effects of *Rhipicephalus* (*Boophilus*) *microplus* salivary gland fractions, agonist and antagonist on platelet adhesion.

4. Conclusion

R. (*B.*) *microplus* tick salivary gland fractions (no. 22, 23, 25, 27, 31, 36, 38, 39 and 51) were found to inhibit thrombin induced platelet aggregation and platelet adhesion to collagen through inhibition of GP1b, GPVI and integrin α2β1 receptors. However, it needs further exploration for potential role in inhibition of haemostasis and thrombosis. The results could aid in identification of candidate peptide for development of anti-tick vaccine and anti-thrombotic drugs.

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