Chapter 3

HEMOSTATIC INTERFERENCE OF KING COBRA (*OPHIOPHAGUS HANNAH*) VENOM: COMPARATIVE CHARACTERIZATION WITH THREE OTHER SNAKE VENOMS OF THE SUBCONTINENT
Chapter 3
Introduction

Russell’s viper (Daboia/Vipera russellii), Saw-scaled viper (Echis carinatus), krait (Bungarus caerulus) and cobra (Naja naja) are endemic to the Indian subcontinent, hence are popularly called ‘big fours’. Therefore, poisonous bite in this region is generally attributed by any one of these snakes. However, the king cobra (Ophiophagus hannah) which is the biggest poisonous snake is confined to thick forest regions of the Western and Eastern parts of the subcontinent and dwell usually away from the human habitat. Lack of therapeutic antivenom for O. hannah venom in the subcontinent supports the previous statement. Because of deforestation and encroachments in recent years, we tend to see king cobra entering in to human territory. At this stage the fact that underscores is that, unlike antivenom therapy available for the big fours, the antivenom therapy is not available to treat the bites of king cobra. In order to get prepared in this line for the possible future threat, it is important to comparatively characterize king cobra venom with the venoms of big fours for biological activities. Systemic manifestations such as neurotoxicity and cytotoxicity are typical of the Elapidae snakes (Lu et al., 2005), while venoms of Viperidae so far are considered to be a rich source of components that primarily affect hemostasis, targeting blood coagulation and platelet function (kamiguti et al., 1998). Components which affect hemostasis have been isolated and characterized from Indian snake venoms. Several proteases (Jagadeesha et al., 2002, Raghavendra gowda et al., 2006, Mahadeswaraswamy et al., 2009, Kumar et al., 2009), factor X activators (Jayanthi et al., 1990) Phospholipase A2 having platelet aggregation inhibiting property (Prasad et al., 1996; Kempbaraju et al., 1999; Rudrammaji et al., 2001; Satish et al., 2004; Kumar et al., 2011) have been reported from the Indian subcontinent.

Though, a non-enzymatic fibrinolytic hexapeptide component “hannapep” from king cobra (Ophiophagus hannah) venom having fibrino(gen)lytic and plasma anti-clotting activity (Gomes et al., 1999) has been reported, comparative evaluation of the O. hannah venom with the whole venoms of the endemic snakes such as D. russellii, E.
carinatus and N. naja have not been studied for their interference in hemostasis. Hemostasis is a complex process and includes the pathways of blood coagulation which results in fibrin clot formation, fibrin clot dissolution and platelet functions (activation or inhibition). Therefore, this chapter systematically compares the hemostatic effects of O. hannah venom with that of the effects of D. russelli, E. carinatus and N. naja venoms and the results are presented.

Materials and Methods

The pooled desiccated Indian King cobra (Ophiophagus hannah) venom was purchased from Mr. Dipak Kumar Mitra, Hindustan Park, Kolkata, India, pooled desiccated venoms of Indian cobra (Naja naja), Russel’s viper (Vipera russelli) and Saw-scaled viper (Echis carinatus) were purchased from Irula snake park, Tamilnadu, India. Fibrinogen (from human plasma, fraction I), Thrombin (from human plasma), Epinephrine and Adenosine diphosphate were purchased from Sigma chemicals Company, St. Louis, USA. Molecular weight markers were purchased from Genei Private Limited, Bangalore, India. Urokinase was from Polamin Werk GmbH, Herdecke, Germany. Uniplastin, Liquicelin and Fibroquant were purchased from Tulip Diagnostics Private limited, Goa, India. All other chemicals and reagents used were of analytical grade. Fresh blood samples were collected from healthy voluntary human donors. Swiss Wister albino mice weighing 18–20 g were from the central animal house facility, Department of Studies in Zoology, University of Mysore, Mysore, India. Animal care and handling were carried out in accordance with the National Regulation for Animal Research.

SDS- Polyacrylamide gel electrophoresis

SDS-PAGE was carried out according to the method of Laemmli, (1970) under reduced and non-reduced condition. Venom samples were prepared with non reducing sample buffer (4% SDS, 2% glycerol, with 0.125 M Tris HCL buffer pH 6.8) and warmed in hot water bath for 3-5 min. 10 µl of the protein Molecular weight marker sample was taken and was mixed with equal volume of reducing sample buffer (Tris-
HCl 0.125M, pH 6.8, 4% SDS, 10% β-mercaptaethanol, 20% Glycerol and 0.02% Bromophenol blue) and was boiled for 1 min. The samples were cooled to room temperature and loaded into the wells. The electrophoresis was carried out using Tris - Glycine buffer (0.25 M Tris and 0.192 M Glycine pH 8.3 containing 0.1 % SDS) at a constant current of 100 V for 2 hrs. The gels were stained for protein bands with Coomassie brilliant blue R – 250 (0.1% w/v) and was destained using methanol, acetic acid and water in the ratio (3:1:6) for 2 hours.

Plasma re-calcification time

The plasma re-calcification time was determined as described by Quick et al., (1935). Four venom samples in various amounts (2-12 µg for D. russellii and N. naja, 0.5-5µg for E. carinatus and O. hannah) were pre-incubated with 0.2 ml of citrated human plasma in the presence of 10 mM Tris-HCl (20 µl) buffer pH 7.4 for 1 min at 37°C. 20µl of 0.25 M CaCl₂ was added to the pre-incubated mixture and clotting time was recorded.

Activated partial thromboplastin time (APTT) and Prothrombin time (PT)

Briefly, 100 µl of normal citrated human plasma and 10 µl of four venoms were pre-incubated for 3 min for dose dependent (2-12 µg) activity. In APTT, the mixture was activated with 100 µl of APTT reagent (LIQUICELIN-E—phospholipids preparation derived from rabbit brain with ellagic acid) for 3 min at 37°C, there after 100 µl of 0.02 M CaCl₂ was added and the clotting time was measured. In case of PT, the clotting time was measured after adding 200 µl of PT reagent (UNIPLASTIN—rabbit brain thromboplastin). The APTT ratio and the International normalized ratio (INR) for PT at each point were calculated from the values of control plasma incubated with the buffer for identical period of time.

Thrombin clotting time (TCT)

Thrombin clotting time was determined according to the slightly modified method of Evans. Briefly, 100 µl of fibrinogen solution (2 mg/ml)/human citrated plasma were pre-incubated with whole venoms of V. russellii, E. carinatus, N. naja and
*O. hannah* in 10 mM Tris-HCl buffer pH 7.4 for varied amount (2-12 µg) for 10 min at 37°C. The clotting time was then determined after the addition of 100 µl diluted thrombin (2.5 NIH units/ml) to the 100 µl of incubation sample.

**Fibrinogenolytic activity**

Fibrinogenolytic activity was tested according to Gao et al., (1998) by incubating venom sample (0.5 µg to 10 µg) with human plasminogen-free fibrinogen (50µg) in 50mM Tris-HCl buffer, 0.1M NaCl, pH 7.4. Both dose dependent and time dependent activities of the venom sample were analyzed. For dose dependent activity, the samples were incubated for 2 hr at 37°C and for time dependent activity samples were incubated at 37°C with different incubation time varying from 0 to 120 min. The reaction was stopped by adding 20 µl of denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptaethanol. The samples were boiled for 5 min, the fibrinogen degradation products were then analyzed on 10% SDS-PAGE.

**Bleeding time**

The bleeding time was assayed by the method of Denis et al., (1998). Briefly, various concentrations (0 to 12 µg) of four venoms in 25 µl of PBS were injected *i.v.* through the tail vein of a group of five mice. After 20 min, mice were anaesthetized using diethyl ether and a sharp cut of 3 mm length at the tail tip of mice was made. Immediately, the tail was vertically immersed into PBS which was pre-warmed to 37°C. Bleeding time was recorded from the time bleeding started till it completely stopped.

**Estimation of fibrinogen concentration**

The four venoms of varied concentration (ranging from 3-12 µg) in 100 µl PBS was injected *i.v.* through the tail vein in to a group of five mice in individual experiments. After 2h the animals were anaesthetized with diethyl ether and the blood samples were collected independently by cardiac puncture using tri-sodium citrate as anticoagulant. The blood samples were centrifuged at 3,000g for 10 min and the plasma
were used for the estimation of fibrinogen according to the protocol provided by the FIBROQUANT kit manufacturer (Tulip Diagnostics Private Limited., Mumbai, India).

**Defibrinogenating Activity**

Defibrinogenating activity was assayed according to the method of Loria et al., (2003). Male albino mice (20g) were injected i.v. with four whole venoms/saline for experimental/control groups in individual experiments. After an hour, blood was collected in test tubes (75×12 mm) by cardiac puncture and the clotting time was recorded. The minimum defibrinogenating dose (MDD) was defined as the minimum amount of venom which, when injected i.v. into male albino mice produced incoagulable blood 1 hr later.

**Fibrinolytic activity**

**Colorimetric estimation**

Plasma clot hydrolyzing activity was assayed according to the method described by Rajesh et al., (2005). Briefly, 100 µl of citrated human plasma/blood was mixed with 30 µl of 0.2 M CaCl₂ and incubated for 2 h at 37°C. The plasma/blood clot obtained was washed thoroughly for 5-6 times with phosphate buffer saline (PBS) and suspended in 400 µl of 0.2 M Tris-HCl buffer pH 8.5. The reaction was initiated by adding varied amounts of four venoms (5-25 µg) in 100 µl of saline and incubated for 2 h 30 min at 37°C. The undigested clot was precipitated by adding 750 µl of 0.44 M trichloroacetic acid (TCA) and allowed to stand for 30 min and centrifuged for 15 min at 1.500xg. The aliquots of 0.5 ml supernatant was transferred to clean glass tubes followed by the addition of 1.25 ml of 0.4 M sodium carbonate and 0.25 ml of 1:3 diluted Folin & Ciocalteu’s phenol (FC) reagent. The color developed was read at 660 nm after allowed to stand for 30 min. One unit of activity is defined as the amount of enzyme required to increase in absorbance of 0.01 at 660 nm/h at 37°C.

**Fibrinopeptides banding pattern**

The method described by Rajesh et al., (2005) was followed with slight modification. The washed plasma clot obtained as described above were suspended in 40 µl of 10 mM Tris-HCl buffer pH 7.4 containing 10 mM NaCl, and 0.05% sodium
azide was incubated with 5µg each of four venoms for 15 h at 37°C. The reaction was terminated by adding 20 µl of sample buffer containing 4% β-mercaptoethanol and 1 M urea and were boiled for 5 min and centrifuged at 5,000xg for 10 min. An aliquot of 20 µl supernatant from each sample was used to analyze the cleavage pattern by 10% SDS-PAGE under reduced condition.

**Plasmin like activity**

The plasminogen activation assay was done according to the method described by Chakrabarty et al., (2000). Briefly, 100 µg each of snake venom in 100 µl of 100 mM potassium phosphate buffer pH 7.4 were independently mixed with 500 µl of azocasein (0.25% in 100 mM potassium phosphate buffer pH 7.4) and incubated for 3 h at 37°C. Addition of 400 µl of 25% trichloroacetic acid abolished the activity. The mixture was centrifuged at 1000 x g for 15 min. The supernatant (600 µl) was diluted with an equal volume 0.5 N NaOH and absorbance was read at 440 nm. One unit of activity was defined as the amount enzyme yielding an increase in absorbance of 0.01/h at 440 nm.

**Platelet aggregation**

Turbidometric method of Born and Cross, (1963) was followed, using a dual channel Chrono-log model 700-2 aggregometer (Havertown, USA). Briefly, 235 µl of PRP suspension was maintained at 37°C in a siliconized glass cuvette and pre-incubated with different doses of the four snake venoms (D. russelli, E. carinatus, N. naja and O. hannah) for 3 min, and the aggregation was initiated by adding the agonist. The agonists such as ADP (10 µM), Epinephrine (10 µM) and Thrombin (1 U/ml) were used in independent experiments. The aggregation was followed for 6 min with constant stirring at the speed of 1200 rpm. In each case, aggregation induced by agonist alone was considered as 100% aggregation. The aggregation trace was the plot of light transmission between platelet rich plasma (PRP) and platelet poor plasma (PPP) baseline, which represent 0% and 100% aggregation respectively.

**Protein estimation**
Protein concentration of venom samples was determined as described by Lowry et al., (1951) using bovine serum albumin (BSA) as standard.

**Statistical analysis**

All the quantitative values were presented as Mean ± SD using SPSS software.

**Results**

Electrophoresis of the four whole venoms in SDS-PAGE under non-reduced condition revealed distinct protein banding patterns that lie in the wide molecular mass range from 3.5 kD to 205 kD. High and low molecular mass proteins are distinct in *O. hannah* venom (Fig. 3.1).

The *D. russellii* and *O. hannah* venoms reduced the recalcification time of citrated human plasma dose dependently while, *N. naja* venom prolonged the recalcification time (Fig. 3.2A). In contrast, *E. carinatus* venom although reduced the clotting time dose dependently, the effect was found to be independent of the added calcium ions as it induced clotting in the absence of calcium ions (Fig. 3.2B).

Similar trend was also observed when tested for APTT where, *N. naja* venom showed prolonged APTT while, the other two venoms decreased the APTT. The effect was moderate in case of *O. hannah* venom while, it was pronounced in case of *D. russellii* venom (Fig. 3.3A). The *N. naja* venom although did not affect much, there observed a slight increased PT, while *O. hannah* venom decreased PT moderately and this effect was pronounced in case of *D. russellii* venom (Fig. 3.3B). By and large the venoms showed similar trend on TCT also where, *N. naja* venom prolonged and the other three venoms reduced the TCT (Fig. 3.3C).

All the four venoms showed proteolytic activity on fibrinogen but with a varied degree and specificity of action. The *D. russellii* venom showed preference for the Aα chain over the Bβ chain while, *E. carinatus* venom showed less preference for the Aα chain but, extensively hydrolyzed both Bβ and γ chains, in contrast both *N. naja* and *O. hannah* venoms preferentially degraded the Bβ chain over Aα chain of fibrinogen. However, the fact that underscores is the resistance of γ chain to the proteolytic
degradation by all venoms except for *E. carinatus* venom (Fig. 3.4) which hydrolyzed it readily.

*In vivo* experiments following independent intravenous injection of venoms through the caudal vein inhibited coagulation of blood in mice as evidenced by continued bleeding resulted in increased bleeding time in all the cases. However, the extent of bleeding varied greatly with the varied doses of venoms injected (Table. 3.1). Further, the defibrinogenation assay involving fibrinogen estimation in the blood of experimental mice revealed the MDD of 6 µg, 3.5 µg, 8.5 µg and 10 µg respectively for *D. russelli*, *E. carinatus*, *N. naja* and *O. hannah* venoms injected independently per 20 g body weight in each case. The fibrinogen content was not detected in blood for 24 hours when administered MDD of individual venoms as compared with the PBS injected control mice which showed fibrinogen content of 242 ± 15 mg/dl.

The fibrinolytic activity of venoms was tested by various methods such as electrophoresis and colorimetric methods. All four venoms degraded the plasma clot but, to a varied extent. The quantitative colorimetric estimation of washed blood clot and plasma clot hydrolysis by all four venoms showed positive results with the *E. carinatus* and *D. russelli* venoms exhibiting highest and least activity respectively while, the other two falls in between (Fig. 3.5). Further, the hydrolysis of individual components of fibrin clot was confirmed by the SDS-PAGE method under reduced condition. The α-polymer and γ-γ-dimers were resistant to hydrolysis except for *E. carinatus* venom. The α-chain was hydrolyzed by both *D. russelli* and *O. hannah* venoms while, the β-chain was exclusively hydrolyzed by *E. carinatus* venom (Fig. 3.6). *N. naja* venom partially degraded α-polymer and α chain. Further, all venoms except *N. naja* venom, hydrolyzed azocasein and the activity measurement by colorimetric method varied as *E. carinatus* > *O. hannah* > *D. russelli* venoms. However, none of the four venoms exhibited plasminogen activation property (Table. 3.2).

All four venoms interfered in human platelet function. *D. russelli* and *N. naja* venoms did not interfere in thrombin induced aggregation in platelet rich plasma while,
O. hannah venom caused >90% inhibition of aggregation (Fig. 3.7A). The ADP induced aggregation was inhibited as D. russellii (95 %) > N. naja (68 %) > O. hannah (36 %) venoms while, The epinephrine induced aggregation was inhibited as D. russellii (94%) > O. hannah (92%) > N. naja (85%). In contrast, the E. carinatus venom by itself caused the clotting of platelet rich plasma. The IC₅₀ values determined for the three venoms for their inhibitory activity on agonist induced platelet aggregation in PRP is given in table 3.3. Based on the IC₅₀ values, the potency of platelet aggregation inhibition varied as D. russellii venom > N. naja venom > O.hannah venom.

Discussion

The present study describes the role of O. hannah, N. naja, D. russellii, and E. carinatus venoms on plasma clot formation, clot dissolution and platelet activation and aggregation functions using in vitro and in vivo assays. The SDS-PAGE banding pattern brings out the possible variations of these venoms as a function of varied protein composition. Blood/plasma coagulation is an acute phase response to vascular injury. The intrinsic (contact activation) pathway and extrinsic (tissue factor) pathway of coagulation both culminate at the site of activation of factor X to Xa which is the initiation site of the common pathway that results in the proteolytic conversion of fibrinogen to fibrin, by thrombin enzyme. Thus, the soluble fibrinogen molecule is transformed in to an insoluble clot. The O. hannah, D. russellii and E. carinatus venoms were found to be pro-coagulant in property as they decreased the citrated plasma recalcification time while, N. naja venom was found to be anticoagulant in property as it prolonged the plasma recalcification time. The results of the plasma recalcification time are in good agreement with the already reported results of D. russellii, E. carinatus and N. naja venoms.

Activated Partial Thromboplastin Time (APTT) and Pro-thrombin Time (PT) respectively are the measures of the efficacy/defects of the intrinsic/common pathways and extrinsic/common pathways of blood/plasma coagulation system respectively. O. hannah and D. russellii venoms by decreasing both APTT and PT appear to exert their
procoagulant activity by activating one or more factors present in both intrinsic and extrinsic pathways of coagulation cascade. Several reports support the above mentioned activities of these venoms in this regard. Carinactivase-1, a Group B prothrombin activator from *E. carinatus* venom requires Ca$^{2+}$ for the activation. RVVX, a metalloprotease and RVV-V, a serine protease from *D. russelli* venom activates factor X and V respectively. A 62 kDa serine protease from *O. hannah* venom is found to activate factor X in a Ca$^{2+}$ dependent manner.

In contrast, *N. naja* venom by increasing APTT and not altering PT appear to act through the intrinsic pathway. The thrombin clotting time (TCT) is a measure of the direct action of thrombin on fibrinogen to generate fibrin, in contrast to the other three venoms which showed decreased TCT, the *N. naja* venom showed increased TCT. All the four venoms degraded fibrinogen and released truncated fibrin. However, the fibrin that was formed due to *N. naja* venom activity failed to polymerize to form the clot and it could probably be due to the degradation of polymerization site located at the C-terminal end while, the other three venoms appear to cleave the fibrinogen from the N-terminal end. However, human plasma when incubated in the absence of calcium with higher amounts of all the venoms did not induce clot formation except for *E. carinatus* venom. Thus, *E. carinatus* venom exhibited thrombin like activity. Release of fibrinopeptides A and or B from the N-terminal end of Aα-chain and/ or Bβ-chain of fibrinogen is the general mechanism of action of thrombin like enzymes. Ecarin, a P-III class metalloprotease, from *E. carinatus* venom is a group A prothrombin activator that activates prothrombin in the absence of calcium, phospholipids and factor V to form the clot. Thus *E. carinatus* venom possesses a battery of procoagulant enzymes.

All the four venoms cleaved the fibrin clot. Similar activity was seen both with washed and unwashed fibrin clot. Though all the four venoms cleaved the fibrin clot, the extent of their fibrinolytic activity varied. This was well exemplified in the SDS banding patterns of the hydrolyzed clot which showed varied preferences of the venoms towards the fibrin bands and colorimetric quantifications of the activities which also varied as *E. carinatus* > *O. hannah* > *D. russelli* > *N. naja*. Thus, all these venoms
showed fibrin(ogen)olytic activity acting both on fibrinogen and as well as fibrin. In vivo experiments involving defibrinogenation and bleeding time assays supported the observed fibrin(ogen)olytic activity of venoms.

A wide variety of venom components can act as procoagulants, causing in vivo activation of the coagulation system, but usually not associated with massive thrombosis and consequent embolic disease, but rather causes consumption of coagulation factors, resulting in anticoagulation. Absence of detectable fibrinogen is due to the procoagulant or anticoagulant activity of the venom components. In addition, the bleeding time associated with all the venoms could also be due to the cleavage of platelet receptors and dissolution of the formed clot. Fibrin(ogen)olytic proteases have been isolated and characterized from Russell’s viper and cobra venoms.

However in addition, except N. naja venom, the other three venoms directly hydrolyzed azocasein. Azocaseinolytic activity in the presence of plasma is the measure of plasminogen activation by venoms where, the formed plasmin degrades the azocasein. However, all the four venoms O. hannah, D. russelli, E. carinatus and N. naja were devoid of plasminogen activation property, the three venoms except N. naja directly acted upon and hydrolyzed azocasein thereby substantiating their plasmin like activity.

Varied inhibitory activities were exhibited when agonists induced platelet aggregation was tested in vitro in the presence of these venoms. The fact that the inhibition of thrombin induced aggregation of platelets by O. hannah venom is possibly be due to the interference of the venom components with the thrombin receptors. There are four types of thrombin receptors which are the Protease Activated Receptors (PARs) PAR1 to PAR4 and they belong to G-protein coupled serpentine family receptors. Of these receptors, PAR1 and PAR4 are the major functional receptors in vivo where PAR1 works at low concentrations of thrombin while PAR4 works at high concentrations of thrombin. However, in in vitro conditions, PAR4 is the major receptor participating. Therefore, O. hannah venom components appear to work either by masking the receptors or by their proteolytic inactivation, hence no more interaction
of the thrombin and this could be the reason for the observed inhibition. While, lack of inhibition in presence of D. russellii and N. naja venoms appear to suggests the absence of agents which interfere with thrombin receptors in these venoms. In support, no report so far claims the studies on the role of thrombin-like activity on platelet aggregation process from D. russellii venom. Similarly, D. russellii, O. hannah and N. naja venoms showed significant of over 80% inhibition of epinephrine induced aggregation of human platelets.

The varied but partial inhibition of ADP induced aggregation by O. hannah and N. naja venoms suggests the presence of agents which either interfere differentially with the ADP receptors (P2 receptors) or the venom components might affect the threshold concentrations of ADP that is required for aggregation. The metalloprotease and PLA\textsubscript{2} enzymes isolated from N. naja venom have been found to cause partial inhibition of ADP induced aggregation. However, so far no component from D. russellii venom has been shown to interfere in platelet function. In contrast, to the other venoms studied, the fact that underscores is that, E. carinatus venom has been found to cause clotting of the PRP before the added agonists. Since, this study has been conducted using PRP, presence of fibrinogen and its cleavage by Ecarin, the thrombin-like enzyme is probably responsible for the observed characteristic PRP clot. Echicetin-a heterodimeric protein from the venom of the Indian saw-scaled viper (Echis carinatus) is reported to bind to platelet glycoprotein Ib (GPIb) and inhibits platelet aggregation.
Figures and Tables:

Fig. 3.1: SDS-PAGE pattern of four venoms: Lanes 1, 2, 3 and 4 respectively represents the banding pattern of 75 µg each of *O. hannah*, *D. russellii*, *E. carinatus* and *N. naja* venoms under non-reduced condition. M represents mol. wt. markers (kD); rabbit muscle myosin (205), phosphorylase B (97.4), Bovine serum albimn (66), ovalbumin (43), carbonic anhydrase (29), soyabeen trypsin inhibitor (20.1), lysozyme (14.3), aprotinin (6.5), insulin (3.5) under reduced condition.
Fig. 3.2A: Effect of venoms on plasma recalcification time: Citrated human plasma, 0.2 ml was incubated with different amounts (0 to 12 µg) of venoms for 5 min at 37°C. Clotting was initiated by adding 20µl of 0.25M CaCl₂ and clotting time was recorded in min. Clotting time recorded in the absence of venoms served as control. Values represent mean ± SD of three independent experiments, P < 0.01.
**Fig. 3.2B: Effect of *E. carinatus* venom on plasma clotting time:** Citrated human plasma, 0.2 ml was added with different amounts (0.02 to 0.2 µg) of *E. carinatus* venom and the clot time was recorded in sec. Values represent mean ± SD of three independent experiments, P < 0.01.
Fig. 3.3: Dose dependent effect of four venoms on the clotting time of normal human citrated plasma: Graphs showing activated partial thromboplastin time ratio (A) for three venoms (D. russelli, N. naja and O. hannah), prothrombin time (B) for three venoms (D. russelli, N. naja and O. hannah) and thrombin clotting time (C) for four venoms (D. russelli, E. carinatus, N. naja and O. hannah). Venom alone in case of E. carinatus could initiate the clotting process in both APTT and PT experiments (data not shown).
Fig. 3.4: Fibrinogenolytic activity of venoms: Lane 1: Fibrinogen (50 μg) alone, lane 2, 3, 4 and 5 containing fibrinogen (50 μg) + 4 μg of *D. russellii*, *E. carinatus*, *N. naja* and *O. hannah* venoms respectively, incubated for 5 h and separated on a 10% SDS-PAGE under reduced condition. Lane M represents molecular weight markers (ranging from 205 to 3.5 kDa) under reduced condition.
<table>
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<th>Venom</th>
<th>Venom in µg</th>
<th>Bleeding time (in min)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>5.25 ± 0.12</td>
</tr>
<tr>
<td>D. russellii</td>
<td>2</td>
<td>7 ± 0.35</td>
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<tr>
<td></td>
<td>4</td>
<td>10 ± 0.40</td>
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<td></td>
<td>5</td>
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<tr>
<td>E. carinatus</td>
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<td>6 ± 0.56</td>
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<tr>
<td></td>
<td>1</td>
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<tr>
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<td>10 ± 0.35</td>
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<tr>
<td></td>
<td>2</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>N. naja</td>
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</tr>
<tr>
<td></td>
<td>4</td>
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<tr>
<td></td>
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<td>10 ± 0.48</td>
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<tr>
<td></td>
<td>7</td>
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</tr>
<tr>
<td>O. hannah</td>
<td>2</td>
<td>6 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>4</td>
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**Table 3.1: Effect of four venoms on the bleeding time in mouse model:** The tail bleeding time in mice models were measured 10 min after intravenous administration of PBS/various doses of four different snake venoms. The values tabulated above are the mean of the values of three independent tests performed ± SD, *P* < 0.01. Bleeding time longer than 10 min was expressed as >10 min.
Fig. 3.5: Fibrinolytic activity of snake venoms by colorimetric method: The washed whole blood clot (A) and plasma clot (B) were incubated independently with increasing amount (25–100 μg) of four snake venoms in 0.2 M Tris-HCl buffer pH 8.5 at 37°C for 2 h 30 min in a total reaction mixture of 500 μl and was further processed as described in the materials and methods.
**Fig. 3.6: Effect of venoms on washed plasma clot:** Washed plasma clot was incubated independently with 5 µg each of *D. russelli*, *E. carinatus*, *N. naja* and *O. hannah* venoms (lane 2, 3, 4 and 5 respectively) for 15 h in 40 µl 10mM Tris-HCl buffer pH 7.4 at 37°C. The reaction was stopped by adding 20 µl denaturing buffer, heated for 3-5 mins in a boiling water bath and were analyzed on a 10% SDS-PAGE under reduced condition. Lane M represents Molecular weight markers in kDa.
Table 3.2: Plasmin-like activity of four venoms.

*One unit of activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01/h at 440 nm.
Fig. 3.7: Inhibitory activities of venoms on platelet aggregation induced by ADP, Epinephrine and Thrombin: Venoms at concentrations ranging from 0 to 40 μg were pre-incubated with PRP for 5 min at 37°C. Platelet aggregation was induced separately by the addition of (A) ADP (10 μM), (B) Epinephrine (10 μM) and (C) Thrombin (1U/ml) and was recorded using a dual channel Chrono-log model 700-2 aggregometer. Values represent mean ± SEM of three experiments.
Table 3.3: IC\textsubscript{50} values for *D. russelli*, *N. naja* and *O. hannah* venoms for their inhibition on agonists induced platelet aggregation in PRP.

IC\textsubscript{50} is defined as the concentration of inhibitor responsible for 50% inhibition of platelet aggregation.

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<tr>
<th>Agonists</th>
<th>IC\textsubscript{50} values in μg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>D. russelli</em></td>
</tr>
<tr>
<td>ADP</td>
<td>4.55</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>4.52</td>
</tr>
<tr>
<td>Thrombin</td>
<td>-</td>
</tr>
</tbody>
</table>