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Hemostatic Interference of Indian King Cobra (*Ophiophagus hannah*) Venom. Comparison with Three Other Snake Venoms of the Subcontinent

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Abstract—Unlike *Naja naja*, *Bungarus caeruleus*, *Echis carinatus*, and *Daboia/Vipera russellii* venoms, *Ophiophagus hannah* venom is medically ignored in the Indian subcontinent. Being the biggest poisonous snake, *O. hannah* has been presumed to inject several lethal doses of venom in a single bite. Lack of therapeutic antivenom to *O. hannah* bite in India makes any attempt to save the victim a difficult exercise. This study was initiated to compare *O. hannah* venom with the above said venoms for possible interference in hemostasis. *Ophiophagus hannah* venom was found to actively interfere in hemostatic stages such as fibrin clot formation, platelet activation/aggregation, and fibrin clot dissolution. It decreased partial thromboplastin time (aPTT), prothrombin time (PT), and thrombin clotting time (TCT). These activities are similar to that shown by *E. carinatus* and *D. russellii* venoms, and thus *O. hannah* venom was found to exert procoagulant activity through the common pathway of blood coagulation, while *N. naja* venom increased aPTT and TCT but not PT, and hence it was found to exert anticoagulant activity through the intrinsic pathway. Venoms of *O. hannah*, *E. carinatus*, and *D. russellii* lack plasminogen activation property as they do not hydrolyze azocasein, while they all show plasmin-like activity by degrading the fibrin clot. Although *N. naja* venom did not degrade azocasein, unlike other venoms, it showed feeble plasmin-like activity on fibrin clot. Venom of *E. carinatus* induced clotting of human platelet rich plasma (PRP), while the other three venoms interfered in agonist-induced platelet aggregation in PRP. Venom of *O. hannah* least inhibited the ADP induced platelet aggregation as compared to *D. russellii* and *N. naja* venoms. All these three venoms showed complete inhibition of epinephrine-induced aggregation at varied doses. However, *O. hannah* venom was unique in inhibiting thrombin induced aggregation.

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Snake venoms are predominantly concentrated mixtures of protein and peptide toxins that possess diverse but specific biological activity. Snakebite is a serious threat that remains as one of the major public health problems, particularly in tropical and subtropical countries. Although the true incidence of snakebite is difficult to estimate, there are about 5 million people affected worldwide each year [1]. The annual snakebite incidence in the Indian subcontinent is more than 2,000,000, and 35,000 to 50,000 of them were fatal [2].

Daboia/Vipera russellii (Russell's viper), *Echis carinatus* (saw-scaled viper), *Bungarus caeruleus* (krait), and *Naja naja* (cobra) are endemic to the Indian subcontinent, and hence they are popularly called the "big four". Therefore, poisonous bite in this region is generally attributed to any one of these snakes. *Ophiophagus hannah* (king cobra), which is the biggest poisonous snake, is confined to thick forest regions of the Western and Eastern parts of the subcontinent and usually dwell away from the human habitat. Because of deforestation and encroachments in recent years, we tend to see *O. hannah* entering into human territory. Unlike antivenom therapy available for the big four, antivenom therapy is not available to treat bites of *O. hannah*. To become prepared for the possible future threat, it is important to comparative-

Abbreviations: aPTT, activated partial thromboplastin time; PRP, platelet rich plasma; PT, prothrombin time; TCT, thrombin clotting time.

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ly characterize *O. hannah* venom with the venoms of the big four for biological activities. Systemic manifestations such as neurotoxicity and cytotoxicity are typical of the Elapidae snakes [3], 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 [4]. Hemostasis is a complex process and includes pathways of blood coagulation that result in fibrin clot formation, fibrin clot dissolution, and platelet functions (activation or inhibition). Several proteases [5-8], factor X activators [9], and phospholipase A_2 having platelet aggregation inhibiting property [10-13] have been reported from the Indian subcontinent.

Components that affect hemostasis have been isolated and characterized from Indian snake venoms. Though a fibrinolytic hexapeptide component "hannapep" from *O. hannah* venom having fibrino(geno)lytic and plasma anti-clotting activity [14] has been reported, comparative evaluation of the *O. hannah* venom with the whole venoms of the endemic snakes for their interference in hemostasis has not been performed. Therefore, the present study was undertaken to systematically compare the hemostatic effects of *O. hannah* venom with those of *D. russellii*, *E. carinatus*, and *N. naja* venoms.

MATERIALS AND METHODS

Pooled desiccated *O. hannah* venom was purchased from Mr. Dipak Kumar Mitra, Hindustan Park, Kolkata, India. The pooled desiccated venoms of *N. naja*, *D. russellii*, and *E. carinatus* were purchased from Irula Snake Park, Tamilnadu, India.

Fibrinogen (from human plasma, fraction I), thrombin (from human plasma), epinephrine, and ADP were purchased from Sigma (USA). Molecular weight markers were purchased from Genei Private Ltd. (India). Urokinase was from Polamin Werk GmbH (Germany). Uniplastin, Liquicelin, and Fibroquant were purchased from Tulip Diagnostics Private Ltd. (India). All other chemicals used were of analytical grade.

Fresh blood samples were collected from healthy voluntary human donors.

Swiss Wistar 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 [15] under reducing and non-reducing conditions. Venom samples were prepared with non-reducing sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 2% glycerol) and warmed in a hot water bath for 3-5 min. Molecular weight marker sample was mixed with equal volume of reducing sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 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 voltage of 100 V for 2 h. The gel was stained for protein bands with 0.1% Coomassie brilliant blue R-250 and was destained using methanol, acetic acid, and water in the ratio 3 : 1 : 6 for 2 h.

Plasma recalcification time. The plasma recalcification time was determined as described by Quick [16]. Four venom samples of various amounts (2-12 μg for *D. russellii*, *N. naja*, and *O. hannah* and 0.02-0.2 μg for *E. carinatus*) were preincubated with 0.2 ml of citrated human plasma in the presence of 10 mM Tris-HCl buffer, pH 7.4, for 1 min at 37°C, and then 20 μl of 0.25 M $CaCl_2$ was added to the preincubated 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 preincubated 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, and thereafter 100 μl of 0.02 M $CaCl_2$ was added and the clotting time was measured. In the 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 an identical period of time.

Thrombin clotting time (TCT). Thrombin clotting time was determined according to the slightly modified method of Evans [17]. Briefly, 100 μl of fibrinogen solution (2 mg/ml) were preincubated with varied amounts (2-12 μg) of whole venoms of *D. russellii*, *E. carinatus*, *N. naja*, and *O. hannah* in 10 mM Tris-HCl buffer, pH 7.4, for 10 min at 37°C. The clotting time was then determined after the addition of 100 μl of diluted thrombin (2.5 NIH units/ml) to 100 μl of the incubation sample.

Fibrinogenolytic activity. Fibrinogenolytic activity was tested according to Gao et al. [18] by incubating venom sample (0.5 to 10 μg) with human plasminogen-free fibrinogen (50 μg) in 40 μl of 50 mM Tris-HCl buffer, 0.1 M NaCl, pH 7.4. Both dose-dependent and time-dependent activities of the venom sample were analyzed at 37°C. For dose-dependent activity, the samples were incubated for 2 h, and for time-dependent activity the incubation time varied 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-mercaptoethanol. The samples were boiled for 5 min, and the fibrinogen degradation products were then analyzed by SDS-PAGE on 10% polyacrylamide gel.

Bleeding time. The bleeding time was assayed by the method of Denis et al. [19]. Briefly, various amounts (0 to 12 μg) of four venoms in 25 μl of phosphate buffered saline (PBS) were injected *i.v.* through the tail vein to a group of five mice. After 20 min, the mice were anaesthetized using diethyl ether and a sharp cut of 3 mm length at the tail tip of the mice was made. Immediately, the tail was vertically immersed into PBS, which was preheated to 37°C. Bleeding time was recorded from the time bleeding started till it completely stopped.

Estimation of fibrinogen concentration. Varied amounts of the venoms (ranging from 3-12 μg) in 100 μl PBS were injected *i.v.* through the tail vein to a group of five mice for each venom. After 2 h, the animals were anaesthetized with diethyl ether, and blood samples were collected independently by cardiac puncture using trisodium citrate as anticoagulant. The blood samples were centrifuged at 3000*g* for 10 min and the plasma samples were used for estimation of fibrinogen according to the protocol provided by the FIBROQUANT kit (Tulip Diagnostics Private Ltd., India).

Defibrinogenating activity. Defibrinogenating activity was assayed according to the method of Loria et al. [20]. Male albino mice (20 g) were injected *i.v.* with the four whole venoms/saline for experimental/control groups in individual experiments. After 1 h, blood was collected into 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 h later.

Fibrinolytic activity (plate method). The method described by Gene et al. [21] was followed with slight modification. A mixture consisting of 2 ml healthy human plasma and 3 ml of 1.2% molten agarose (45°C) in 10 mM Tris-HCl buffer, pH 7.4, containing 0.02% sodium azide, 70 mM $(NH_4)_2SO_4$, 90 mM NaCl, 0.70 mM $MgCl_2$, and 200 μl of 0.2 M $CaCl_2$ was poured into 10 × 90 mm Petri dish and was allowed to solidify for 2 h at 27°C. Venom samples (10 μg) and 1 IU of urokinase in 10 μl of 10 mM Tris-HCl buffer, pH 7.4, were placed independently on the surface of the gel matrix and incubated for 15 h at 27°C. The diameter of the lysed zones was measured and photographed.

Fibrinolytic activity (colorimetric estimation). Plasma clot-hydrolyzing activity was assayed according to the method described by Rajesh et al. [22]. Briefly, 0.1 ml of citrated human plasma/blood was mixed with 30 μl of 0.2 M $CaCl_2$ and incubated for 2 h at 37°C. The plasma/blood clot obtained was washed thoroughly 5-6 times with PBS and suspended in 0.4 ml of 0.2 M Tris-HCl buffer, pH 8.5. The reaction was initiated by adding varied amounts of the four venoms (5-25 μg) in 0.1 ml of saline and incubated for 2.5 h at 37°C. The undigested clot was precipitated by adding 0.75 ml of 0.44 M trichloroacetic acid (TCA) and allowed to stand for 30 min and then centrifuged for 15 min at 1500*g*. Aliquots of 0.5 ml supernatant were 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 and Ciocalteu's phenol reagent. The color developed was read at 660 nm after standing for 30 min. One unit of activity is defined as the amount of enzyme required to increase in absorbance at 660 nm by 0.01/h at 37°C.

Fibrinopeptide banding pattern. The method described by Rajesh et al. [22] was followed with slight modification. Washed plasma clots 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 and incubated with 5 μg each of the 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 the samples were boiled for 5 min and centrifuged at 5000*g* for 10 min. An aliquot of 20 μl supernatant from each sample was used to analyze the cleaved protein banding pattern by SDS-PAGE in 10% polyacrylamide gel under the reduced conditions.

Plasminogen activation/plasmin-like activity. The plasminogen activation assay was done according to the method described by Chakrabarty et al. [23]. Briefly, 100 μg of each venom sample in 0.1 ml of 100 mM sodium phosphate buffer, pH 7.4, was incubated with 0.5 ml of azocasein (0.25% in 100 mM sodium phosphate buffer, pH 7.4) in presence and absence of 20 μl of human plasma for 3 h at 37°C. Addition of 0.4 ml of 25% TCA abolished the activity. The mixture was centrifuged at 1000*g* for 15 min. The supernatant (0.6 ml) was diluted with an equal volume of 0.5 N NaOH, and absorbance was read at 440 nm. Azocasein incubated with plasma or urokinase served as negative control experiments. One unit of activity was defined as the amount enzyme yielding an increase in absorbance at 440 nm of 0.01/h.

Platelet aggregation. The turbidimetric method of Born and Cross [24] was followed using a dual channel Chrono-log model 700-2 aggregometer (Havertown, USA). Briefly, 235 μl of platelet rich plasma (PRP) suspension was maintained at 37°C in a siliconized glass cuvette and preincubated with different doses of the four snake venoms for 3 min, and the aggregation was initiated by adding the agonist (10 μM ADP, 10 μM epinephrine, or 1 U/ml thrombin). Aggregation was followed for 6 min with constant stirring at a speed of 1200 rpm. In each case, the aggregation induced by the agonist alone was considered as 100% aggregation. The aggregation trace was the plot of light transmission between PRP and platelet poor plasma (PPP) base line, which represent 0 and 100% aggregation, respectively.

Protein concentration of the venom samples was determined as described by Lowry et al. [25] using bovine serum albumin (BSA) as the standard. All the quantitative values were presented as mean ± SD using SPSS Sigmastat software.

RESULTS

SDS-PAGE analysis. Electrophoresis of the four whole venoms in SDS-polyacrylamide gel under non-reducing condition revealed distinct protein banding patterns that lie in a wide molecular mass range from 3.5 to 205 kDa. High- and low-molecular-mass proteins are distinct in the *O. hannah* venom (Fig. 1).

Plasma coagulation, aPTT, PT, and TCT. 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. 2). In contrast, although *E. carinatus* venom reduced the clotting time dose-dependently, the effect was found to be independent of added calcium ions as it induced clotting in their absence (data not presented).

A similar trend was observed when tested for aPTT, where *N. naja* venom showed prolonged aPTT while the other two venoms decreased the aPTT. *Daboia russellii* venom reduced the aPTT moderately, and this reduced effect was much more pronounced in the case of *O. hannah* venom (Fig. 3a). Although the *N. naja* venom did not affect PT much, a slightly increased PT was observed,

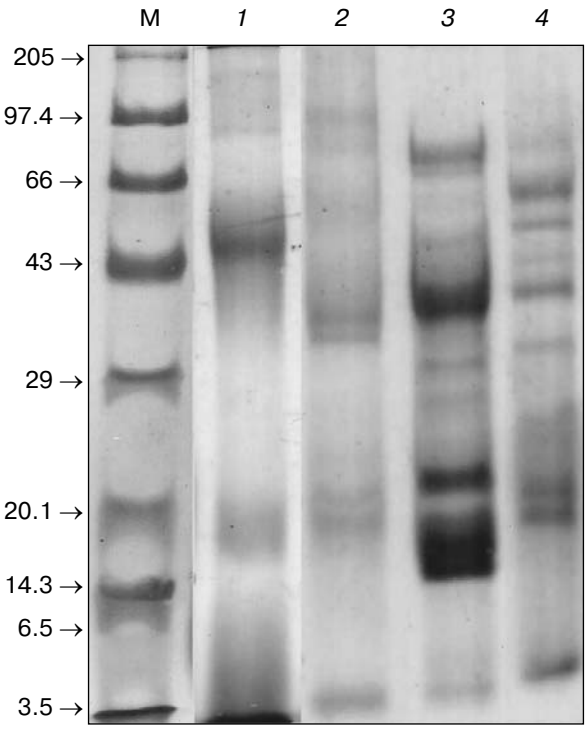

Fig. 1. SDS-PAGE pattern of venoms. Samples *1*, *2*, *3*, and *4* represent the banding pattern of 75 μg each of *O. hannah*, *D. russellii*, *E. carinatus*, and *N. naja* venoms, respectively, under non-reducing conditions. M represents the following markers (kDa): rabbit muscle myosin (205), phosphorylase B (97.4), bovine serum albumin (66), ovalbumin (43), carbonic anhydrase (29), soybean trypsin inhibitor (20.1), lysozyme (14.3), aprotinin (6.5), and insulin (3.5).

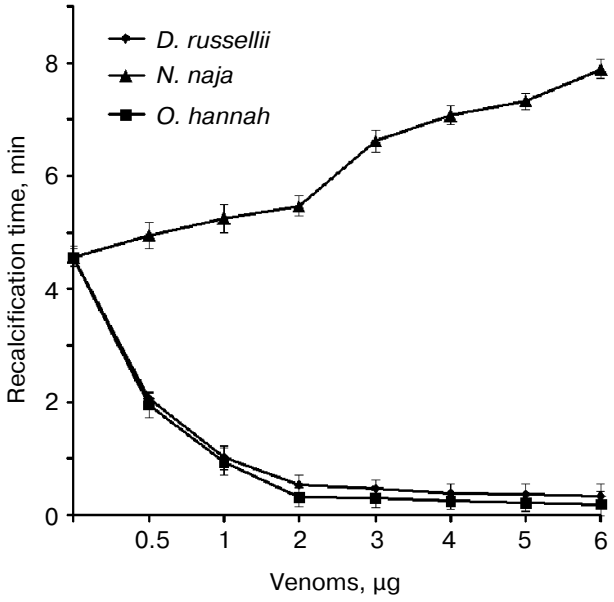

Fig. 2. Effect of venoms on plasma recalcification time.

while both *O. hannah* and *D. russellii* venoms showed significantly decreased PT (Fig. 3b). Further, all four venoms interfered in TCT with *O. hannah*, *D. russellii*, and *E. carinatus* venoms causing decreased TCT while *N. naja* venom prolonged the TCT (Fig. 3c).

Fibrinogenolytic activity. All 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. The γ chain was resistant to proteolytic degradation by all venoms except for *E. carinatus* venom (Fig. 4).

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 1). Further, the defibrinogenation assay involving fibrinogen estimation in the blood of experimental mice revealed the MDD of 6, 3.5, 8.5, and 10 μg, respectively, for *D. russellii*, *E. carinatus*, *N. naja*, and *O. hannah* venoms injected independently per 20 g body weight in each case.

Fibrinolytic activity. The fibrinolytic activity of venoms was tested by the plate, electrophoretic, and colorimetric methods. All four venoms degraded the plasma clot, but to a varied extent. The individual venoms at 5 μg each varied as *E. carinatus* > *O. hannah* > *N. naja* > *D. russellii* venoms with the respective activity zones of 12 ± 0.8, 10 ± 0.6, 7 ± 0.6, and 5 ± 0.8 mm in a semiquantitative

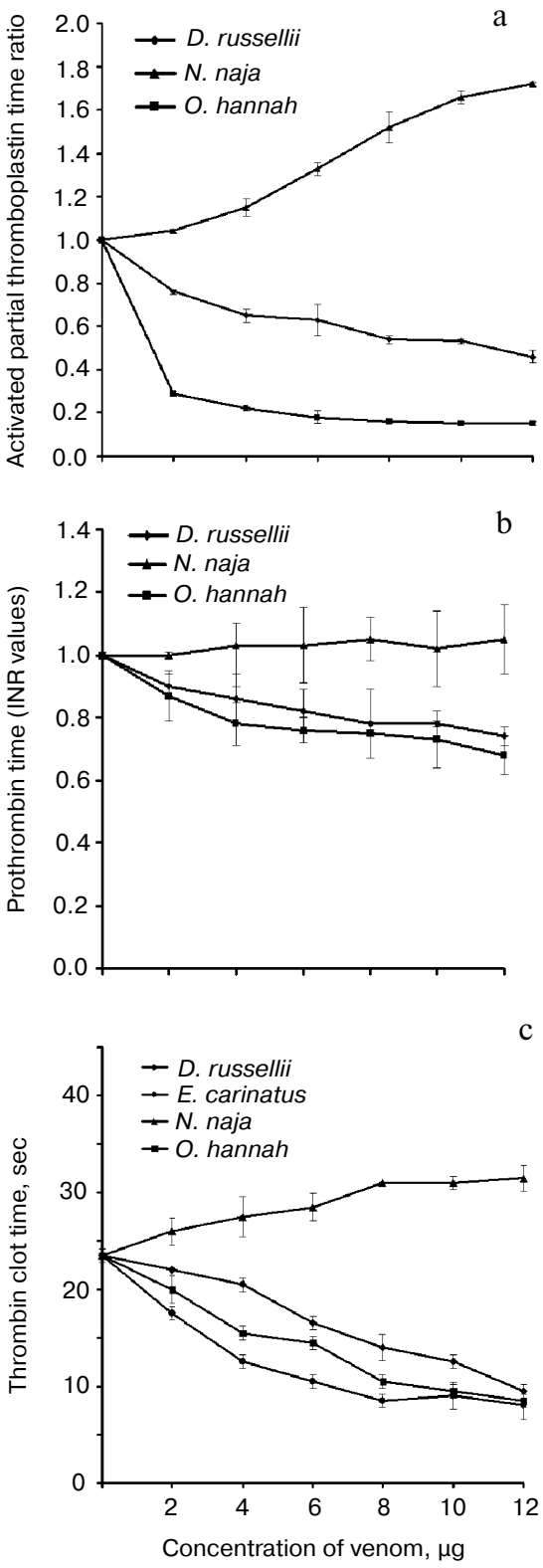

Fig. 3. Dose-dependent effects of four venoms on the clotting time of normal human citrate-treated plasma. a) Activated partial thromboplastin time ratio; b) prothrombin time; c) thrombin clotting time. Venom alone in case of *E. carinatus* could initiate the clotting process in both aPTT and PT experiments (not shown).

agarose gel plate method, while urokinase revealed the activity zone of 9 ± 0.6 mm for 1 IU, which served as the positive control (figure not shown). The dose-dependent quantitative colorimetric estimation of plasma clot hydrolysis by all four venoms is also consistent with the plate method. The activity varied as *E. carinatus* venom > *O. hannah* venom > *D. russellii* venom > *N. naja* venoms. Thus, *E. carinatus* venom was most active and *N. naja* venom was least active on plasma clot (Fig. 5). Further, the hydrolysis of individual components of fibrin clot was confirmed by the SDS-PAGE method under reducing conditions. The α polymer and γ–γ dimers were resistant to hydrolysis except for by *E. carinatus* venom. The α chain was hydrolyzed by both *D. russellii* and *O. hannah* venoms, while the β chain was exclusively hydrolyzed by *E. carinatus* venom. *Naja naja* venom partially degraded α polymer and α chain (Fig. 6). Further, all venoms except *N. naja* venom hydrolyzed azocasein, and the activity measured by the colorimetric method varied as *E. carinatus* > *O. hannah* > *D. russellii* venoms. However, none of the venoms exhibited plasminogen activation property (Table 2).

Platelet aggregation. All four venoms interfered with human platelet function. Venoms of *D. russellii* and *N. naja* did not interfere in thrombin-induced aggregation in platelet rich plasma, while *O. hannah* venom caused >90% inhibition of aggregation (Fig. 7c). The ADP-induced aggregation was inhibited as *D. russellii* (95%) > *N. naja* (68%) > *O. hannah* (36%) venoms (Fig. 7a), while the epinephrine-induced aggregation was inhibited as *D. russellii* (94%) > *O. hannah* (92%) > *N. naja* (85%) (Fig. 7b). In contrast, the *E. carinatus* venom by itself caused

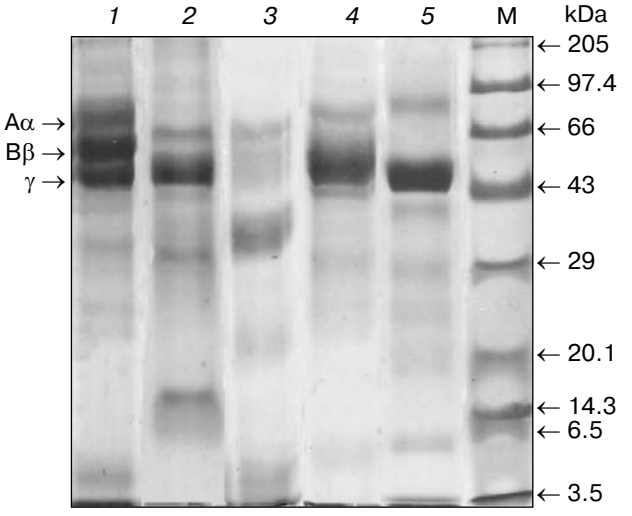

Fig. 4. Fibrinogenolytic activity of venoms. Lanes: *1*) fibrinogen (50 μg); *2-5*) fibrinogen (50 μg) + 4 μg of *D. russellii*, *E. carinatus*, *N. naja*, or *O. hannah* venom, respectively; 2-h incubation. The samples were separated on a 10% SDS-polyacrylamide gel under reducing conditions. Lane M represents molecular weight markers under reducing conditions.

Table 1. Effect of four venoms on bleeding time in mouse model

Venom	Venom amount, μg	Bleeding time, min
Control	0	5.2 ± 0.1
D. russellii	2	7.0 ± 0.4
	4	10.0 ± 0.4
	5	>10
E. carinatus	0.5	6.0 ± 0.6
	1	8.0 ± 0.4
	1.5	10.0 ± 0.4
	2	>10
N. naja	2	6.0 ± 0.4
	4	8.0 ± 0.3
	6	10.0 ± 0.5
	7	>10
O. hannah	2	6.0 ± 0.2
	4	8.0 ± 0.4
	5	10.0 ± 0.3
	6	>10

the clotting of PRP. Based on the IC_{50} values, the potency of platelet aggregation inhibition varied as *D. russellii* venom > *N. naja* venom > *O. hannah* venom (data not presented).

DISCUSSION

The present study describes the effects 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 reveals 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. Thus, the soluble fibrinogen molecule is transformed into an insoluble clot. The *O. hannah*, *D. russellii*, and *E. carinatus* venoms were found to be procoagulant agents 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 for *D. russellii*, *E. carinatus*, and *N. naja* venoms [7, 8, 26]. Interestingly, *O. hannah* venom is an Elapid venom, but it shares great similarity with Viperid venoms in this respect.

aPTT and PT are measures of the efficacy/defects of the intrinsic/common pathways and extrinsic/common pathways of blood/plasma coagulation system, respectively. Venoms of *O. hannah* and *D. russellii*, 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 the coagulation cascade. Several reports support the above-mentioned activities of these venoms. Carinactivase-1, a group B prothrombin activator from *E. carinatus* venom, requires Ca^{2+} for the activation [27]. Metalloprotease RVV-X and serine protease RVV-V from *D. russellii* venom activate factors X and V, respectively [28, 29]. A 62-kDa serine protease from *O. hannah* venom is found to activate factor X in a calcium-dependent manner [30].

In contrast, *N. naja* venom, by increasing aPTT and not altering PT, appears to act through the intrinsic pathway. The 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. The venoms degraded fibrinogen and released truncated fibrin. However, the fibrin that was formed due to *N. naja* venom activity failed to polymerize to form a 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 α chain and/or β chain of fibrinogen is the general mechanism of action of thrombin-like enzymes. Ecarin, a P-

Table 2. Plasmin-like activity of four venoms

Sample	Activity, U/h	
	without human plasma	with human plasma
Azocasein	<1.0	<1.0
Azocasein + urokinase (200 IU)	<1.0	2.4 ± 0.2
Azocasein + *D. russellii* venom (100 μg)	5.6 ± 0.2	5.6 ± 0.2
Azocasein + *E. carinatus* venom (100 μg)	12.2 ± 0.2	12.8 ± 0.4
Azocasein + *N. naja* venom (100 μg)	<1.0	<1.0
Azocasein + *O. hannah* venom (100 μg)	9.5 ± 0.3	9.7 ± 0.4

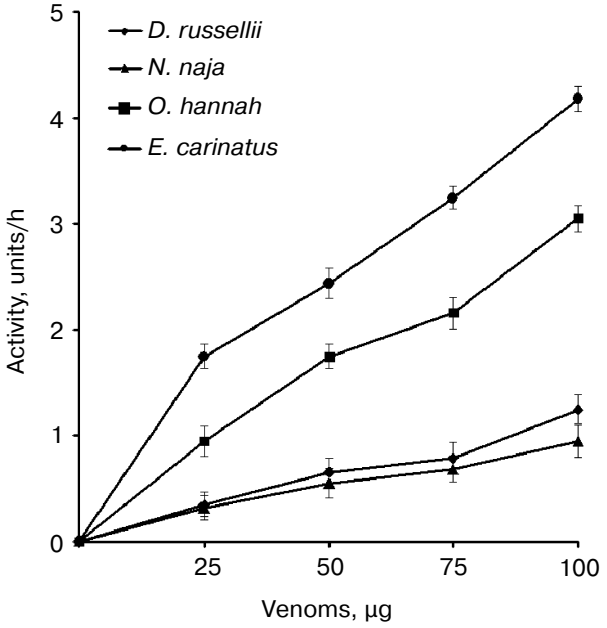

Fig. 5. Fibrinolytic activity of snake venoms by the colorimetric method.

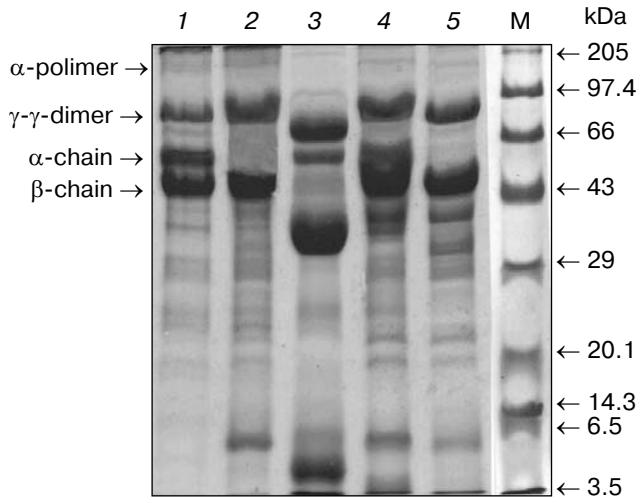

Fig. 6. Effect of venoms on fibrinolytic activity: washed plasma clot was incubated in the absence (*1*) or presence of 5 μg of *D. russellii* (*2*), *E. carinatus* (*3*), *N. naja* (*4*), and *O. hannah* (*5*) venoms for 15 h in 40 μl of 10 mM Tris-HCl buffer, pH 7.4, at 37°C. The reaction was stopped by adding 20 μl denaturing buffer, and the samples were heated for 3-5 min in a boiling water bath and analyzed on a 10% SDS-polyacrylamide gel under reducing conditions. Lane M represents molecular weight markers.

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 [31]. Thus *E. carinatus* venom possesses a battery of procoagulant enzymes.

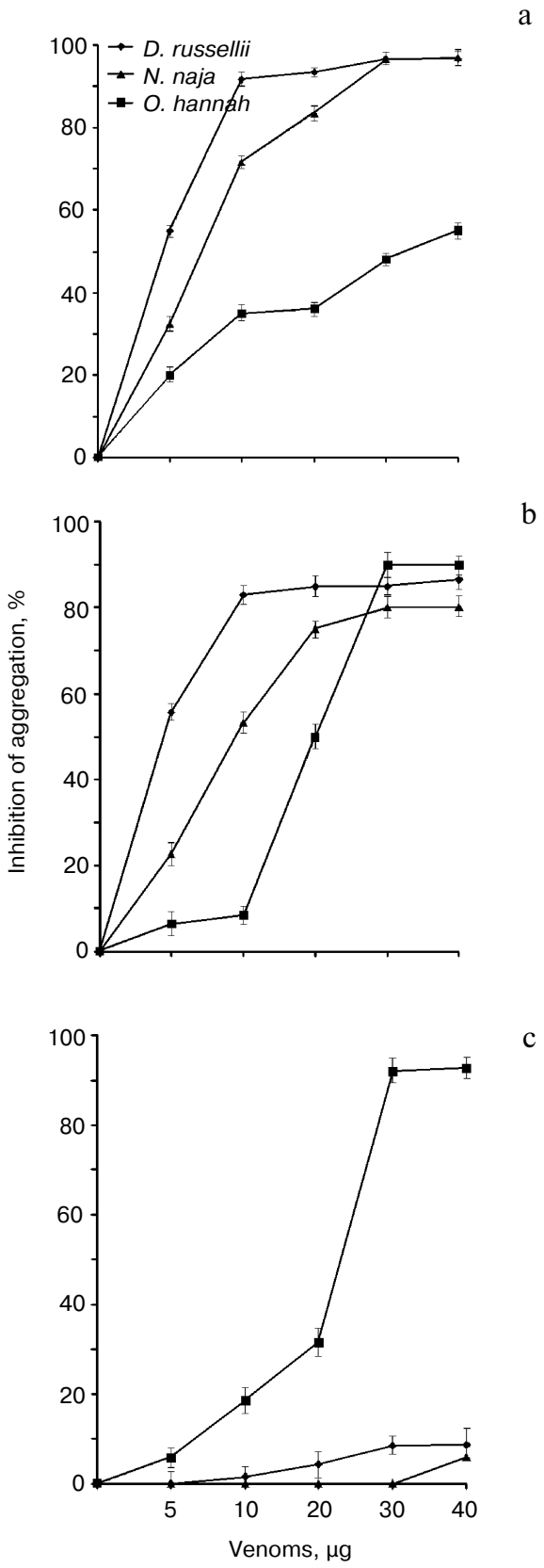

Fig. 7. Inhibitory activities of venoms on platelet aggregation induced by ADP (a), epinephrine (b), and thrombin (c).

All the venoms cleaved a fibrin clot. Similar activity was seen both with washed and unwashed fibrin clot. However, the extent of their fibrinolytic activity varied. This was well exemplified in the plate method where all the venoms formed clear lysed zones but of varied diameter. Furthermore, the SDS banding patterns of the hydrolyzed clot showed varied preferences of the venoms towards the fibrin bands, and colorimetric quantifications of the activities indicated that they varied as *E. carinatus* > *O. hannah* > *D. russellii* > *N. naja*. Thus, all these venoms showed fibrin(ogen)olytic activity acting both on fibrinogen and fibrin. *In vivo* experiments involving defibrinogenation and bleeding time assays supported the observed fibrin(ogen)olytic activity of the 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. Fibrino(geno)lytic proteases have been isolated and characterized from Russell's viper and cobra venoms [8, 32].

In addition, except *N. naja* venom, the three other venoms hydrolyzed azocasein. Azocaseinolytic activity in the presence of plasma is a measure of plasminogen activation by venoms, where the formed plasmin degrades the azocasein. However, the venoms of *O. hannah*, *D. russellii*, *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 agonist-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 due to the interference of the venom components with thrombin receptors. There are four types of thrombin receptors, which are the protease-activated receptors 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, under *in vitro* conditions PAR4 is the major receptor participating. *Ophiophagus hannah* venom components may inhibit either by masking the receptors or by their proteolytic inactivation, canceling their interaction with thrombin. The lack of inhibition in the presence of *D. russellii* and *N. naja* venoms appears to suggest the absence of agents that interfere with thrombin receptors in these venoms. In support, no report so far claims the role of thrombin-like activity in platelet aggregation process from *D. russellii* venom. Similarly, *D. russellii*, *O. hannah*, and *N. naja* venoms showed significant (>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 that 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 [33] and PLA_2 enzymes isolated from *N. naja* venom [12, 13, 34] 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 with platelet function. In contrast to *O. hannah*, *D. russellii*, and *N. naja* venoms, *E. carinatus* venom has been found to cause the aggregation of PRP independent of the added agonists. Since this study has been conducted using PRP, the presence of fibrinogen and its cleavage by ecarin, a 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 (*E. carinatus*), is reported to bind to platelet glycoprotein Ib (GPIb) and inhibit platelet aggregation [35].

In conclusion, *O. hannah* venom shows similarities with *N. naja* venom in fibrinogen degradation. However, though an Elapid, *O. hannah* venom shares similarities with the Viperid venom with respect to procoagulant activity, plasmin-like activity, and inhibition of platelet aggregation induced by ADP and epinephrine. Although *O. hannah* can deliver several lethal doses of venom in a single bite, it is highly likely that early treatment can save the victim. Obviously, this requires better understanding of the king cobra venom pharmacology in relation to the pharmacology of known venoms in order to make therapeutic antivenom.

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