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RESEARCH ARTICLE

Biochemical and biological characterization of *Naja kaouthia* venom from North-East India and its neutralization by polyvalent antivenom

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ABSTRACT

This study describes biochemical and biological properties of *Naja kaouthia* (Indian monocled cobra) venom of North-East India. The LD_{50} of the crude venom was found to be 0.148mg/kg and neurotoxicitic symptoms like paralysis of lower limbs and heavy difficulty in breathing at sub-lethal dose in mice was observed. The venom exhibited PLA_2, indirect hemolytic and myotoxic activities but showed weak proteolytic and low direct hemolytic activities. It did not exhibit any hemorrhage when injected intradermally to mice. Anticoagulant activity was prominent when recalcification, prothrombin and activated partial thrombinplastin time were tested on platelet poor plasma. Rotem analysis of whole citrated blood in presence of venom showed delay in coagulation time and clot formation time. Fibrinogen of whole citrated blood was depleted by venom when analyzed in Sonoclot. Crude venom at 10µg and after 16hr of incubation was found to degrade α chain of fibrinogen. Neutralization study showed that Indian polyvalent antivenom could neutralize some of the biochemical and biological activities as well as its fibrinogenolytic activity.

KEYWORDS: *Naja kaouthia*, haemostasis, thromboelastometry, myotoxicity, polyvalent antivenom

INTRODUCTION

Snakebite envenoming is a neglected tropical disease (WHO), which requires immediate attention. It is estimated that globally 2.5 million people are bitten by snakes each year with ~85,000 deaths (Gutierrez et al, 2010); in India, approximately 35,000 to 40,000 people die of snakebites annually (Chippaux, 1998; Kasturiratne et al, 2008). According to recent National Mortality Survey data, the incidence of snakebite cases is likely to be more than 50,000 per year in India (Mohapatra et al, 2011). However, these data may be far from the truth as most of the incidences happen in rural areas and these deaths mostly remain unreported. In India the "Big Four", *Naja naja*, *Bungarus caeruleus*, *Daboia russelii* and *Echis carinatus* are considered to be medically important snakes and are responsible for most of the deaths. Recently, it has been reported that hump-nosed pit viper (*Hypnale hypnale*) from Kerala, is capable of causing lethal envenomation (Joseph et al, 2007). Hence, in addition to the "Big Four", there might be other medically important snakes in specific geographical locations, which need attention. This is important for clinical diagnosis for treatment and for production of effective antivenoms. In India, polyvalent antivenom is raised against the "Big Four" venoms but these snakes may not be present throughout the country. Moreover, administration of this polyvalent antivenom has well documented limitations (Offerman et al, 2001; Lalloo and Theakston, 2003; Williams et al, 2007).

Naja kaouthia is recognized phenotypically with the presence of O-shaped or monocellate hood pattern. They are widely distributed in Nepal, North East India, Bangladesh, Myanmar, Thailand and Peninsular Malaysia (Whitaker, 1978; Viravan et al, 1992; Mukherjee and Maity, 2002).

According to WHO, it belongs to Category 1 of venomous snakes. The symptoms of cobra bite are general neurotoxicity leading to flaccid paralysis and death by respiratory failure, and also severe hypertension (Agarwal et al, 2006; Halesha et al, 2013). Symptoms of coagulopathy have also been reported in victims of *Naja kaouthia* of Asian origin (Khandelwal et al, 2007). The *Naja kaouthia* venom of North-East India origin has not been explored though venom of West Bengal (India) origin have been studied extensively (Mukherjee and Maity, 2002; Lalloo and Theakston, 2003; Mukherjee, 2007; Debnath et al, 2010; Sekhar and Chakrabarty, 2011). Hence, some work on biochemical and biological characterization of the *Naja kaouthia* venom and its *in vitro* neutralization by Indian polyvalent antivenom has been undertaken previously.

MATERIALS AND METHODS

Reagents and kits

$sPLA_2$ assay kit was procured from Cayman Chemical Company (MI, USA). NEOPLASTINE® CL PLUS and APTT reagent were obtained from STAGO (France). AGAPEE kit for CK/LDH analysis was purchased from AGAPPE diagnostics (Switzerland), Glass beads gbACT+ kit was obtained from Sienco, Inc. (USA). Polyvalent antivenom manufactured by Bharat Serums and Vaccines Limited (India) was purchased locally. Bovine plasma fibrinogen was obtained from Sigma-Aldrich and all other reagents used were of analytical grade and were either from Merck or Sigma-Aldrich, (USA).

Animals

Male Swiss albino mice of 40±3gm were obtained from central animal facility, University of Mysore. All animal were housed in well ventilated cages and experiments were carried out according to the Animal Ethical Committee Protocol (University of Mysore, Mysore, India, Proposal no. UOM/IAEC/25/2011).

Collection of snake venom, preparation and storage

Adult *Naja kaouthias* were captured from Jamugurihat, district Sonitpur, Assam, North-East India in the, month of May from its natural habitat and venom was extracted by allowing the snake to bite into a sterile beaker covered with para-film. The crude venom was immediately desiccated using dehydrated silica gel and stored in -20°C until further use. The permission for milking of snakes was obtained from Principal Chief Conservator of Forest (Wild Life) and Chief Wild Life Warden of Assam, India (WL/FG.27/tissue Collection/09 dated 07/10/2011).

Determination of protein content

Total protein content of *Naja kaouthia* venom was determined according to Lowry's method using BSA as standard (Lowry et al, 1951).

Phospholipase A_2 (PLA_2) activity

PLA_2 activity was assayed using $sPLA_2$ assay kit according to the manufacturer's protocol (Cayman Chemical Company, MI, USA). Briefly, in a 96-well microtitre plate, 10µl of venom (0.1mg/ml), 10µl DTNB (5, 50-dithio-bis-(2-nitrobenzoic acid)) and 5µl assay buffer were added. The reaction was initiated by adding 200µl of substrate solution (diheptanoyl Thio-PC). After gentle shaking, the optical density was measured every minute at 405nm using MultiSkan GO multi plate reader (Thermo Scientific, USA) for 10min. Assay buffer was used as blank and bee venom PLA_2 (0.01mg/ml) was used as a positive control. Tests were carried out in triplicate and mean values were taken. The activity was expressed as micromoles of diheptanoyl Thiol-PC hydrolyzed per min per mg of enzyme.

Caseinolytic assay

Digestion of casein was evaluated according to the method of Ouyang and Teng (Ouyang and Teng, 1976). Briefly, 1% (w/v) casein in 20mM Tris-Cl, pH 7.4, was incubated with various amounts of venom protein (1, 5, 10, 50 and 100µg) for 1hr at 37°C. Reaction was stopped by addition of ice cold 10% (v/v) TCA and centrifuged for 10min at 5000rpm (Thermo Scientific, USA, Heraeus Multifuge X1R). The digested protein in the supernatant was determined according to Lowry's method (Lowry et al, 1951). Tyrosine curve was used to determine the protease activity and one unit of protease activity is defined as *n* mole equivalent of tyrosine formed per min per ml.

LD_{50} determination

Toxicity of the venom was analyzed according to the method of Meier and Theakston (Meier and Theakston, 1986). Briefly, various amount of freshly dissolved venom (0.05 to 1mg/kg) in saline was injected intraperitoneally to eight male Swiss albino mice in a final volume of 150µl and the controls were injected with saline alone. The animals were carefully monitored for 24hr and their survival time was recorded and LD_{50} was determined.

Edema inducing activity

The procedure of Yamakawa et al, (Yamakawa et al, 1976) as modified by Vishwanath et al, (Vishwanath et al, 1988) was followed. Mice weighing 20–30gm were injected with varying amount of venom (2–15µg) in a total volume of 20µl saline into intra plantar surface of right hind foot pad. Respective left foot pad received 20µl of saline and served as vehicle. Control mice were injected with 20µl saline into intra plantar surface of both hind foot pads. After 45min the mice were anesthetized (barbitone, 30mg/kg, i.p.) before sacrifice and hind limbs were removed at the ankle joint and weighed individually. The increase in weight due to edema is expressed as the ratio of the weight of edematous limb to the weight of vehicle (saline injected) limb x100. The amount of venom required to cause an edema ratio of 120% (20% above the basal level) is defined as minimum edema dose (MED).

Hemorrhagic activity

Hemorrhagic activity was assayed as described by Kondo et al, (Kondo et al, 1960). Various amount of venom (2–15µg) in 30µl saline were injected intradermally into mice and control mice received saline instead of venom sample. After 3hr, mice were sacrificed using anesthesia (barbitone, 30mg/kg, i.p.). The dorsal surface of the skin was removed and the inner surface was observed for hemorrhagic lesions. *E. carinatus* venom was used as positive control. The minimum hemorrhagic dose (MHD) is defined

as the concentration of venom that induce a hemorrhagic spot of 1cm diameter from the spot of injection.

***In-vivo* myotoxicity**

For myotoxicity, release of serum creatine kinase (CK) and lactate dehydrogenase (LDH) in the blood were determined using AGAPPE kit (AGAPPE diagnostics, Switzerland). Group of six male albino mice were injected (i.m) with 15µg crude venom (40µl) and control received 40µl of saline. After 3hr, mice were anesthetized and 0.5ml of blood samples was drawn using cardiac puncture. The serum obtained by centrifugation was diluted with saline at 1:20 ratio. The CK and LDH activity were measured in 10µl of plasma according to the manufacturer's protocol and were expressed in Units/liter (U/l). The results are mean ±SD of three experiments.

Collection of Blood and Platelet Poor Plasma (PPP) preparation

Fresh goat blood was collected in citrated tube (0.11M tri sodium citrate) at 1:9 ratios (citrate: blood) from local butcher's shop. Human blood was collected from healthy donors (27Yr) who had not taken any medication for last 48hr. 9ml of blood was drawn with 20 gauge 3/4" needle and immediately transferred to a plastic tube containing 1ml of 0.11M tri sodium citrate (Suntravat et al, 2010). The tubes were centrifuged at 3000rpm (Thermo Scientific, USA, Heraeus Multifuge X1R) for 15mins to separate the red blood cells (RBC) and platelet poor plasma (PPP) and used within 4hr of collection.

Direct and indirect hemolytic activity

The RBC pellet obtained from the blood (as described above) was washed 4–5 times and re-suspended in 0.9% (w/v) saline to a final concentration of 10% (v/v). Various amount of venom were incubated for 60min at 37°C with 150µl of 10% RBC to a final volume of 2ml with 0.9% (v/v) NaCl. The tubes were centrifugation at 5000rpm (Thermo Scientific, USA, Heraeus Multifuge X1R) for 10min and the absorbance of the supernatant was measured at 540nm in a MultiSkanGO, UV-Vis spectrophotometer (Thermo Scientific, USA). The hemolysis caused by dH_2O was considered as 100%. For Indirect hemolytic, 20µl of egg yolk substrate solution was added to the reaction mixtures at the time of incubation and hemolysis was measured as described for direct hemolytic activity. The results are mean ±SD of three experiments.

Fibrinogenolytic activity

Fibrinogenolytic activity was assayed according to the method of Ouyang and Teng, using bovine fibrinogen (2mg/ml) dissolved in 50mM Tris HCl buffer, pH 7.5, 0.15M NaCl (Ouyang and Teng, 1976). To 300µl of dissolved fibrinogen, various amount of venom in 150µl of buffer was incubated for different time intervals at 37°C. The incubated mixtures were then run on a 12.5% (w/v) SDS-PAGE according to the method of Laemmeli (Laemmli, 1970). Staining was done with 0.25% (w/v) Coomassie brilliant blue R250 and destained till the protein bands were visible.

***In-vitro* coagulant assays**

Recalcification time

Recalcification time of human PPP was measured using coagulation analyzer (STAGO, France). Various amount of venom in 50µl of PBS was pre-incubated with 50µl of human PPP at 37°C for 3min and 50µl of 25mM $CaCl_2$ was added to initiate the clot formation. The clotting time with PBS was considered as normal clotting time. The results are as mean ±SD of three experiments.

Prothrombin time (PT) test

Prothrombin time was measured using PT reagent (NEOPLASTINE® CL PLUS) obtained from STAGO (France) according to the manufacturer's protocol on a coagulation analyzer (STAGO, France). Various amount of venom in 50µl of PBS was pre-incubated with 50µl of human PPP at 37°C for 1min and 100µl of PT reagent was added to initiate the clot formation. The clotting time with PBS was considered as normal clotting time. The results are mean ±SD of three experiments.

Activated partial thrombin time (APTT) test

Activated partial thrombin time was determined using APTT reagent obtained from STAGO (France) according to the manufacturer's protocol on a coagulation analyzer (STAGO, France). Various amount of venom in 50µl PBS was incubated with 50µl of human PPP and 50µl of APTT reagent for 3min at 37°C. The clot formation was initiated by adding 50µl of 25mM $CaCl_2$. The clot formation time with PBS was considered as normal clotting time. The results are mean ±SD of three experiments.

Whole citrated blood analysis

Thromboelastometry analysis

To quantify the CT (clotting time, in seconds), CFT (clot formation time, in seconds) and MCF (maximum clot firmness, in mm) of the whole citrated blood, Rotem® Analyzer (ROTEM®Pentapharm GmbH Diagnostic Division; Munich, Germany) was used. For the analysis, blood samples from healthy volunteers were collected in 0.11M tri sodium citrate at 9:1 (blood: citrate) ratio. Various amount of venom in 20µl of PBS was mixed with 20µl of 200mM $CaCl_2$, to this reaction mixture, 320µl of whole citrated blood was added and clot formation was observed over 30min. Clot formation function with only PBS was considered as control. The results are mean ±SD of three experiments.

Sonoclot analysis

A glass bead activated test tube (gbACT+ Kit obtained from Sienco, Inc, USA) was used to monitor clot detection, clot rate and platelet function (clot retraction) in a Sonoclot Coagulation and Platelet Function Analyzer (Sienco, Inc, USA). Various amount of venom in 20µl of PBS was added to 320µl of citrated human blood followed by 20µl 200mM $CaCl_2$. The head assembly of the analyzer was closed 10s after the start button was pressed. Data were acquired and analyzed with Signature Viewer software (Sienco, Inc.). The results are mean ±SD of three experiments.

Neutralization studies

For neutralization studies, various amount of polyvalent antivenom was pre-incubated with 1µg of *Naja kaouthia* venom in a final volume of 20µl for 1hr at 37°C and assays were performed as described above. The percentage inhibition was calculated by considering the activity in absence of polyvalent antivenom as 100%. The results are mean ±SD of three experiments.

RESULTS

Biological characterization

The biochemical and biological activities of the crude venom are listed in Table 1. The median lethal dose (LD_{50}) was found to be 0.148mg/kg when injected intraperitoneally to experimental mice. When sub-lethal dose of venom was injected to mice, neurotoxic symptoms like difficulty in movement; breathing and frequent drinking of water were observed followed by death after 40min. The amount of CK and LDH released after injection of 15µg of venom was found to be 6.605U/l and 26.38U/l respectively in the plasma. The CK was 10 times more than observed for the control mice (0.63U/l), however, the LDH was found to be only 3U more. The minimum edema dose (MED) of the venom was found to be 11.25µg. No direct hemolytic activity was observed up to 10µg of venom but when the amount was increased up to 100**µg**, it exhibited 1.4% RBC hemolysis. For indirect hemolytic activity, 23% hemolysis was observed for 1µg of venom. The venom showed weak proteolytic activity when tested on casein. The amount of tyrosine liberated was 0.14±0.02 moles by 100µg of venom in 1min. PLA_2 activity of the venom was 7.584µmol/min/mg when assayed using $sPLA_2$ assay kit. However no haemorraghic spot was observed when 3µg of venom was injected intradermally (Figure 1).

In-vitro coagulation activities

The venom showed anticoagulant activity in dose dependent manner. When recalcification time of human plasma was tested with 1µg venom, the plasma did not form clot up to 500s whereas the normal clotting time was 126.5sec (Figure 2). Prothrombin time increased dose dependently and at 0.1µg, clot formation was not observed up to 500sec. The APTT test on plasma did not increase significantly up to 0.1µg but when the amount was increased to 1µg venom the plasma did not form clot (Figure 2).

In Rotem® Analyzer, coagulation time (CT) for 0.1µg venom was 634±15sec and for the control it was observed to be 503±10sec. When the amount of the venom was increased to 1 and 10µg, clot formation was not observed which is depicted by a straight line (Data not shown). The clot formation time (CFT) in presence of 0.1µg of venom was recorded to be 266±10sec, whereas the CFT for control plasma was only 87±3s (Table 2). Maximum clot firmness (MCF) value at 0.1µg of venom was 61±1.3mm, whereas for the control the value was 65±2mm. However, at higher concentration of venom the blood clot did not form. Hence, the values were not measurable in the Rotem® analyzer (Table 2).

The activated clotting time (ACT) increases dose dependently and at 10µg of venom it was recorded to be 591±1.3sec in Sonoclot Coagulation and Platelet Function Analyzer. At 0.1µg venom the clot rate was similar to normal clot rate (normal range 9–35sec) but with increase in concentration, the clot rate decreased which might be due to depletion of fibrinogen. However, up to 1.0µg the platelet function was found to be normal but at 10µg the platelet function was not observed (Table 2). Lower amount of venom did not show any digestion of fibrinogen (data not shown). However, when the amount of venom was increased to 10µg, clear digestion of α chain of fibrinogen was observed after 16hr of incubation (Figure 3A).

Neutralization studies

Effect of polyvalent antivenom on some of the biochemical and biological properties of *Naja kaouthia* venom are shown in Table 3. At 1:1 ratio, the polyvalent antivenom could

Table 1. Some biochemical and biological activities of *Naja kaouthia* venom

Parameters	Activity
LD_{50}	0.148 mg/kg
PLA_2 activity assay	7.9±0.24$^{\Psi}$
Direct hemolytic assay (100µg venom)	1.4±0.51%
Indirect hemolytic assay (1µg venom)	23.0 ±3%
Caseinolytic activity (100µg venom)	0.14±0.02*
Creatine kinase (CK) (15µg i.m. injection)	6.6±0.2 U/l
Lactate dehydrogenase (LDH) (15µg i.m. injection)	26.3±2.3U/l
Minimum edema dose (MED)	11.2±0.18 µg
Haemorraghic activity (up to 15µg)	NA

*Normal CK and LDH values are 0.63 U/l and 23.39 U/l respectively; Ψµmol of diheptanoyl Thiol-PC hydrolyzed/min/mg; **n* moles of tyrosine formed/min; NA= No Activity. Results are mean ±SD (n=3)

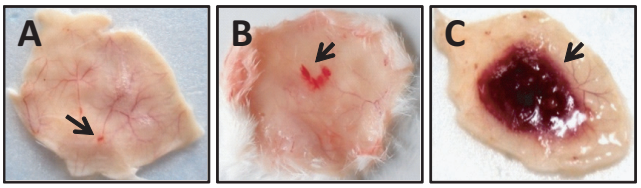

Figure 1. Haemorraghic activity of *Naja kaouthia* venom. **A.** Control (30µl of saline), **B.** *Naja kaouthia* venom (15µg), **C.** Saw scaled viper venom (3µg) (Positive control), the arrow indicates site of injection.

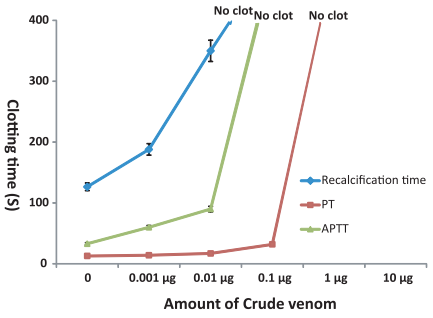

Figure 2. Dose dependent anticoagulant activity of *Naja kaouthia* venom on human plasma. Effect of crude venom on Recalcification time, Prothrombin Time test (PT) and Activated Partial Thrombin Time test (APTT). The results are mean ±SD of three experiments.

Table 2. Anticoagulant activity of *Naja kaouthia* venom on whole citrated blood. Results are expressed as mean ±SD of three experiments.

Parameters	PBS	Crude venom (µg/ml)		
		0.1	1.0	10
Thromboelastometry analysis				
Coagulation time (CT) (s)	503±10	634±15	>1200	>1200
Clot formation time (CFT) (s)	87±3	266±10	NCF	NCF
Maximum clot firmness (MCF) (mm)	65±2	61±1.3	NCF	NCF
Sonoclot analysis				
Activated clotting time (ACT) (s) (range: 128–213)	176±5.2	215±7.4	243±6.3	591±10
Clot rate (CR)(range: 9.0–35)	23±0.5	23±0.32	16±0.21	1.2±0.2
Platelet function (range: 3–5)	2.8±0.01	3.8±0.02	3.3±0.01	0

*NCF: No clot formation, the results are expressed as mean ± SD

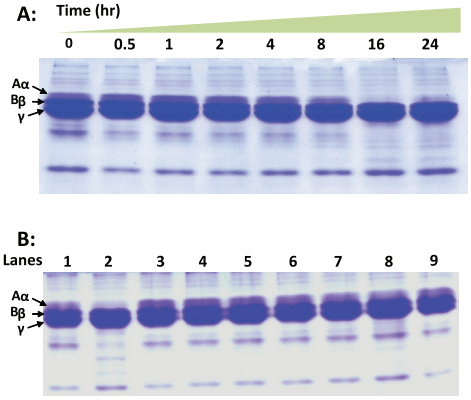

Figure 3. A. Fibrinogenolytic activity of *Naja kaouthia* venom. SDS-PAGE of bovine fibrinogen (reduced) after incubation with 10µg crude *Naja kaouthia* venom at various time intervals. **B.** Inhibition of fibrinogenolytic activity of *Naja kaouthia* by polyvalent antivenom. The venom:polyvalent antivenom (1:1, w/w) mixture was pre-incubated for 1hr at 37°C. This mixture was incubated with 300µl of fibrinogen (2mg/ml) for 24hr and aliquots were withdrawn at different time interval and fractionated in 12.5% (w/v) SDS-PAGE. **Lane 1.** Undigested fibrinogen (control). **Lane 2.** Fibrinogen incubated with only venom; **Lane 3.** After 0.5hr; **Lane 4.** 1hr; **Lane 5.** After 2hr; **Lane 6.** After 4hr; **Lane 7.** After 8hr; **Lane 8.** After 16hr; and **Lane 9.** After 24hr.

not neutralize the PLA_2 activity of the venom but at 1:100 ratios, 97.38 ± 4.8% inhibition was observed. Inhibition of the indirect hemolytic activity of venom was also observed similar to the PLA_2 activity. When the concentration of the polyvalent antivenom was increased by 100 times, indirect hemolytic activity was completely neutralized. Recalcification time of the venom was neutralized up to 49.34% at 1:1 ratio and with 10 times increase in polyvalent antivenom, 92.03% neutralization was observed. Similarly, the APTT and PT was also brought to the normal clotting time when the polyvalent antivenom was 10 times excess of the venom concentration. Moreover, degradation of α chain of fibrinogen by venom was inhibited by polyvalent antivenom at 1:1 ratio (Figure 3B).

DISCUSSION

The patho-physiological effect post-snakebite envenomation varies greatly among the various species and even within species due to variation in the venom proteins and biological activities (Glenn et al, 1983; Minton and Weinstein, 1986; Daltry et al, 1996; Saravia et al, 2002; Menezes et al, 2006). These variations affect the clinical manifestation of envenomation and require specific consideration for treatment. Hence understanding the biochemical and biological properties of snake venom from a particular geographic location is important.

The LD_{50} of the *Naja kaouthia* venom was found to be 0.148mg/kg, whereas those for cobra venoms of Thailand and Kolkata origin were reported to be 0.23mg/kg and 0.7mg/kg, respectively (Mukherjee and Maity, 2002; Leong et al, 2012). Though the route of injection was different (Kolkata origin venom given via tail vein injection) in these experiments, the lethal dose of North East origin venom was less than that of the other geographical locations suggesting it might be more lethal. However, the comparative study with indistinguishable experimental conditions would be necessary to differentiate these venoms. In mice the venom did not induce haemorraghic activity and venom of Kolkata origin is reported to be devoid of such activities. The haemorraghic is mainly caused by metalloproteases, which are abundantly found in viper venom (Kamiguti et al, 1996; Chakrabarty et al, 2000; Mukherjee, 2008). Moreover, the edema inducing activity was not found to be significant. Hence this venom might not induce inflammation and tissue damage at the site of bite. Interestingly, the venom at 100µg showed only 1.4% hemolysis of RBC, whereas at the same amount Kolkata venom activity is reported to be 39.0% (Mukherjee and Maity, 2002). The membrane damaging activity is mainly contributed by the low molecular weight proteins which might be absent in this venom. The indirect hemolytic activity of the venom in presence of the egg yolk is due to PLA_2 enzymes. The lysophospholipids and free fatty acids formed during the catalysis of phospholipids by PLA_2 enzyme exhibits this activity as they are lytic in nature (Condrea et al, 1964). The presence of various PLA_2 isoenzymes and neurotoxins in *Naja kaouthia* venom have been

Table 3. *In vitro* neutralization of whole venom activity by polyvalent antivenom

Activity	% inhibition by polyvalent antivenom		
	1:1	1:10	1:100
PLA_2 activity	0	40.0±5.0	97.38 ± 4.8
Indirect hemolytic	11.96±2.12	68.15±0.15	100
Recalcification time	49.34±5.01	92.03±3.0	96.52±2.81
PT	36.44±5.8	78.19±3.86	99±1.76
APTT	32.33±6.44	92.1±5.83	100
Fibrinogenolytic	α chain present	α chain present	α chain present

*The results are expressed as mean ± SD (n=3)
Values indicate % inhibition at each venom:antivenom (µg:µg) ratio

reported by various workers (Joubert and Taljaard, 1980; Meng et al, 2002; Qiumin et al, 2002; Doley et al, 2004). When the crude venom was analyzed for the PLA_2 activity using diheptanoyl Thiol-PC as substrate, the amount of substrate hydrolyzed product was 7.9±0.24µmol/min/mg suggesting the presence of enzymatically active PLA_2 in the venom. PLA_2 is one of the major constituent in the elapid venom, which confers multiple toxicity to the prey or victim such as membrane damaging, neurotoxicity, edema and prolongation of coagulation time (Kini and Evans, 1989; Doley et al, 2004). Hence the myotoxicity, neurotoxicity and edema induced by this venom are due to the presence of large amount of PLA_2 enzyme in the venom. The observed differences in the biochemical and biological activities in the venoms of Indian origin might be due to variation in the venom composition and content due to difference in geographical locations. Both venoms were collected during summers; however, in the present study, the ages of the snakes were unknown as they were captured from the wild. Detailed analysis of *Naja kaouthia* venoms from different locations of India need to be carried out to decipher the differences in the venom composition as well as the presence of unique toxins.

Snake venom proteins affect the haemostasis process of victim/prey either by prolonging or shortening the clotting time. Elapid venoms are anticoagulant in nature due to the presence of large amount of strong and weak anticoagulant PLA_2 enzymes. Moreover, non-enzymatic protein from elapid venom like Cardiotoxins from *Naja nigricollis crawshawii* and Hemextin A and hemextin AB complex from *Hemachatus haemachatus* venom are also reported to be anticoagulant in nature (Kini et al, 1988; Banerjee et al, 2005). The venom significantly delayed the recalcificaion time, PT and APTT of plasma under *in vitro* condition, which is due to strong anticoagulant proteins present in the venom. The plasma did not form clot at 0.01, 0.1 and 1µg concentration of venom when tested for recalcificaion time, PT and APTT, respectively. This suggests that the anticoagulant activity of the venom is most likely to affect all the pathways. Venom PLA_2 enzymes inhibit activation of FX to FXa which leads to disruption in the formation of prothrombinase complex, which is required for blood coagulation (Stefansson et al, 1990; Kerns et al, 1999; Kini, 2005). The higher amount of venom required in case of PT and APTT for non-coagulation of blood might be due to the addition of extra phospholipids during these tests; however, this needs to be verified. The venom proteins, especially the PLA_2 enzymes, hydrolyze the phospholipids which are required for the prothrombinase complex formation. The Sonoclot and Rotem analysis also demonstrated that the *Naja kaouthia* venom is anticoagulant in nature. The whole citrated blood analysis by sonoclot clearly indicated the depletion of fibrinogen in the reaction when pre-incubated with venom. The lower value of MCF by Rotem analysis indicates decreased platelet number or function, decreased fibrinogen level or fibrin polymerization disorders, or low activity of factor XIII. Recently, Nk a metaloprotease, which cleaves the α- chain, as well as a low molecular protein with fibrin(ogen)olytic activity have been reported (Wijeyewickrema et al, 2007; Debnath et al, 2010). The weak proteolytic activity towards casein and higher amount of venom and time required for complete degradation of α chain of bovine serum fibrinogen might be due to presence of these proteins in lower amount. Hence anticoagulant activity of *Naja kaouthia* might not be only due to degradation of phospholipids or α chain of fibrinogen but action of different venom proteins which might be acting enzymatically or non-enzymatically on coagulation factors and complexes.

Polyvalent antivenom is currently used by the medical practitioners for the treatment of snakebite patients in India. The Indian polyvalent antivenom is prepared using the venoms of four major poisonous snake species viz: *Naja naja, Daboia russelii, Echis carinatus* and *Bungarus caeruleus.* In most of the cases, it has been observed that the efficacy is highly reduced when antivenoms raised against venom from a particular geographic region is used to treat victims from another region (Shashidharamurthy et al, 2002; Shashidharamurthy and Kemparaju, 2007). The polyvalent antivenom could neutralize some of the biochemical and biological activity partially at 1:10 ratio (venom: polyvalent antivenom) and complete neutralization was observed when the dose of the polyvalent antivenom was increased to 10 fold. The partial inhibition might be due to the antibodies of *Naja naja* proteins present in the polyvalent antivenom, which recognizes the *Naja kaouthia* venom proteins. Present study documents that the polyvalent antivenom can neutralize some of tested biochemical and biological activities of *Naja kaouthia* venom under *in vitro* condition.

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

CFT; clot formation time
CT; Coagulation time
MCF; Maximum clot firmness
ACT; Activated clotting time

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